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LOCALIZATION OF GLUCOSE, GLUCONATE, AND GLUCOSE-6-PHOSPHATE OXIDATION SYSTEMS IN EXTRACTS OF PSEUDOMONAS FLUORESCENS[1]

R. G. EAGON

Abstract

Cell-free extracts of *Pseudomonas fluorescens*, strain OSU 64, derived by means of sonic oscillation and alumina grinding, were assayed to determine the localization of enzymes necessary for the oxidation of glucose, gluconate, and glucose-6-phosphate. Glucose and gluconate were oxidized to 2-ketogluconate by both the supernatant and particulate fractions of the extracts. Approximately 40% of the activity for the oxidation of glucose was demonstrated to be in the supernatant fraction and 60% in the particulate fraction. Activity for the oxidation of gluconate was found to be located with approximately 50% in each fraction. Oxidation of glucose-6-phosphate was observed only in the soluble portion.

Introduction

The study of glucose dissimilation with cell-free enzymatic extracts of *Pseudomonas fluorescens* was first reported by Wood and Schwerdt (15, 16, 17). At the same time the author of this paper was carrying on similar experiments (4). The former workers obtained their extracts by means of sonic oscillation while the latter used the method of alumina grinding. The results obtained from these two laboratories compared favorably. There was, however, one noticeable difference. Wood and Schwerdt reported that the non-phosphorylative oxidation systems for glucose and gluconate, which oxidize these compounds to 2-ketogluconate, were associated with a red particulate fraction. Similarly, the oxidation systems for the phosphorylated intermediates, e.g., glucose-6-phosphate, etc., also appeared to be particulate in nature. This writer, on the other hand, observed that systems catalyzing the oxidation of these substrates could be demonstrated in the supernatant even after centrifugation at 125,000 × *g* for 60 minutes.

Owing to the recent emphasis on the localization of enzymes in the microbial cell and, particularly, in cell-free extracts, it was felt that a reinvestigation of this problem was warranted. Furthermore, a survey of the literature revealed

[1]Manuscript received July 29, 1957.
Contribution from the Department of Bacteriology, the University of Georgia, Athens, Georgia, U.S.A. Presented in part at the meeting of the Society of American Bacteriologists held in Detroit, Michigan, U.S.A., 1957.

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little information of value concerning the localization of enzyme systems involved in either the glucose to 2-ketogluconate conversion or the hexose-monophosphate shunt mechanism.

Experimental Methods and Results

Cultivation

Pseudomonas fluorescens, strain OSU 64, was grown aerobically at 30° C. in a mineral-salts medium containing glucose and supplemented with yeast extract, as previously described (5). A rotary-type shaker was employed and 100 ml. of medium were used in 500-ml. Erlenmeyer flasks.

Preparation of Extracts

Extracts were prepared both by sonic oscillation and by alumina grinding. Sonic extracts were prepared in phosphate buffer from washed cells of cultures 18 hours old using a Raytheon 9 kc., 50 watt sonic oscillator. Cells collected from 500 ml. of medium (1.5 g. dry weight) were suspended in 25 ml. of $M/15$ phosphate buffer, pH 6.9–7.0, and oscillated for a period of 2–15 minutes. Periods of oscillation shorter than 2 minutes did not result in sufficient breakage of cells for study. Extracts to be used in the experiments were prepared from the crude sonic extracts by centrifugation for 30 minutes at 7500 $\times$ *g* (at 1.5 cm. from the bottom of the tube). Extracts thus prepared contained approximately 8.4 mg. protein per ml. The extracts were prepared and handled at all times at 4° C.

Conventional techniques of alumina grinding were employed, using Alcoa A303 alumina. Since *P. fluorescens* is quite easy to rupture, grinding with alumina for 2 minutes resulted in a high degree of cell breakage. After grinding, the extracts were prepared in sufficient $M/15$ phosphate buffer, pH 6.9–7.0, to yield the same concentration of protein per ml. as the sonic extracts and handled from this point on in the same manner as for the sonic extracts.

Localization of the Enzyme Systems in the Extract

Extracts, prepared as described, were subjected to differential centrifugation by means of a refrigerated Spinco ultracentrifuge, model L. The fractions thus obtained were assayed for systems catalyzing the non-phosphorylative oxidation of glucose and gluconate and for glucose-6-phosphate oxidation systems. The results expressed in Fig. 1 indicate the localization of the enzymes in the extract and in the supernatant and particulate fractions of the extract after centrifugation at 34,000 $\times$ *g* for 60 minutes. The particulate matter obtained by the centrifugation was resuspended in $M/15$ phosphate buffer, pH 6.9–7.0, in sufficient quantity to yield a volume equal to the volume of the original extract from which it was obtained. It can be seen, then, that the systems oxidizing glucose and gluconate appear in both fractions while the oxidation of glucose-6-phosphate took place only in the supernatant fraction. Furthermore, addition of the cofactors ATP or DPN (1 μM. per Warburg vessel) or such metal activators as magnesium, manganese, zinc,

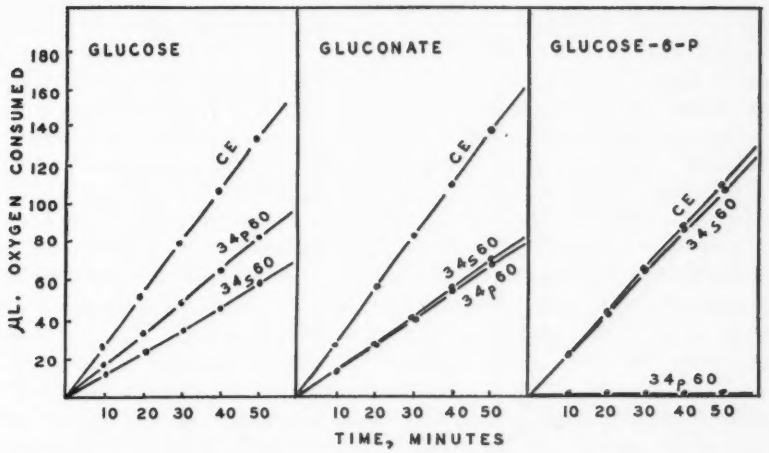

FIG. 1. Localization of enzyme systems catalyzing the oxidation of glucose, gluconate, and glucose-6-phosphate. Each Warburg vessel contained 1.0 ml. extract or fraction thereof, 0.5 ml. 1% substrate, and 0.2 ml. 15% KOH (center well). Endogenous subtracted. CE contained approximately 8.4 mg. protein/ml.; 34_s60—7.6 mg. protein/ml.; 34_p60—0.8 mg. protein/ml.

iron, or calcium (10 μM. per Warburg vessel) did not stimulate oxidation of glucose-6-phosphate by the particulate fraction.

Enzyme activity was estimated by the measurement of the rate of oxygen uptake using a conventional Warburg respirometer under conditions where there would be sufficient substrate to saturate the enzymes being investigated. Protein was determined using a Kjeldahl digestion, and ammonia determination was made with Nessler's reagent. The system used to designate the fractions is the one proposed by Alexander and Wilson (1), and is based on the centrifugal force and time used for their preparation. Thus, the 34_p60 fraction consists of particles ($_p$) which are sedimented at 34,000 $\times$ g in 60 minutes, the prefix indicating the force in 1000 $\times$ g and the suffix referring to the time in minutes.

Although not indicated in Fig. 1, similar results were observed when the extract was centrifuged at 135,000 $\times$ g for as long as 2 hours. On the other hand, the oxidative systems being investigated appeared only in the supernatant fraction when the extract was centrifuged for 60 minutes at 14,000 $\times$ g, indicating that the particulate matter was of such size that it required a centrifugal force greater than this in order to be sedimented.

The data recorded in Table I indicate: (1) the recovery in each fraction of the percentage activity of the extract (% CE) for the oxidation of the indicated substrates, the extract being considered as 100% and activity measured as rate of O_2 uptake per minute; (2) the ratio of the specific activity in the fractions to that in the extract (conc.), the specific activity being the μl. O_2 uptake per minute per mg. protein; and (3) the mg. protein found in each fraction.

TABLE I

PERCENTAGE OF THE ACTIVITY OF THE EXTRACT (% CE), RATIO OF THE SPECIFIC ACTIVITY TO THE CELL EXTRACT (CONC.), AND THE QUANTITY OF PROTEIN FOUND IN EACH FRACTION (MG. P/ML.)*

	34,60			34,60			135,60			135,60		
Substrate	% CE	Conc.	mg.P/ml.	% CE	Conc.	mg.P/ml.	% CE	Conc.	mg.P/ml.	% CE	Conc.	mg.P/ml.
Glucose	46	0.48	7.75	60	4.66	0.88	23	0.34	7.06	57	3.75	1.50
	45	0.46	7.38	70	6.53	0.75	34	0.43	6.81	64	4.77	1.25
Gluconate	50	0.52	7.75	50	4.65	0.88	61	0.90	7.06	43	2.61	1.50
	50	0.56	7.38	50	3.97	0.75	57	0.77	6.81	50	3.46	1.25
Glucose-6-phosphate	100	1.11	7.75	0	0	0.88	89	1.06	7.06	0	0	1.50
	100	1.01	7.38	0	0	0.75	80	0.96	6.81	0	0	1.25

*Results from two different experiments are indicated. The calculations were made as outlined in Fig. 1 and the text.

Inspection of Table I indicates that the sum of the activities (% CE) of the supernatant plus particles expressed for each centrifugal force used and for the oxidation of each substrate equal approximately 100%, i.e., equal to the unfractionated extract. It is also apparent that little change resulted for the oxidation of the substrates by the fractions as the centrifugal force was increased. Furthermore, although nearly twice as much protein was found in the 135_p60 fraction as in the 34_p60 fraction, the results were not significantly altered. This indicates that the enzyme systems associated with the particles were sedimented by a centrifugal force of 34,000 $\times$ g and that an increase in centrifugal force did not result in an appreciable increase of sedimentation.

In some cases, as indicated in Table I, less than 100% recovery of the enzyme systems resulted. This appeared to be due to enzyme inactivation, since recombination of the fractions resulted in approximately the same percentage activity as the sums of the individual fractions.

The results obtained from sonic extracts and alumina-ground extracts appeared to be identical. The results in Table I, however, are those obtained from sonic extracts. It is significant also that the same pattern of localization was found regardless of the period of oscillation (varying from 2 to 15 minutes) when the extracts were studied as just described.

Identification by Chromatography of 2-Ketogluconate as the End Product of the Non-phosphorylative Oxidation of Glucose and Gluconate

It was observed that 1 μl. O_2 was taken up per molecule of glucose and 1/2 μl. O_2 per molecule of gluconate when these substrates were oxidized by either the extract or the supernatant or particulate fractions. This is the quantity of oxygen required to oxidize these compounds to 2-ketogluconate and this reaction has been well established by other workers (12, 15, 16). Protein was precipitated from the contents of the Warburg reaction vessels by the addition of not less than one volume of acetone and the resulting supernate reduced in volume *in vacuo* over P_2O_5. Chromatographic prodecures, as described by Norris and Campbell (10), using a solvent composed of methanol, ethanol, and water (45:45:10 by volume) and an ammoniacal silver nitrate spray reagent were employed. Only one spot was observed with an R_f value of approximately 0.45. This is theoretical for 2-ketogluconate. Furthermore, 2-ketogluconate, used as a control, was found to have an identical movement.

Cyanide Inhibition Studies

The enzyme systems involved in the non-phosphorylative oxidation of glucose and gluconate by either the extract or the supernatant or particulate fractions were demonstrated to have an inhibition of only about 80% in the presence of 0.001 M potassium cyanide. In view of the fact that the fractions studied contained small amounts of protein (see Table I), this would indicate that the systems involved were relatively insensitive to cyanide inhibition. Furthermore, examination of the reaction mixtures during oxidation of these substrates with a Leitz hand spectroscope revealed the appearance of absorption bands at approximately 550 mμ, indicating the participation of

cytochrome *c*. Absorption was not detected in the region of cytochrome oxidase (600–620 mμ). This has been reported before (4, 15, 16). The spectroscopic evidence, along with the relative insensitivity to cyanide, has been interpreted by Wood and Schwerdt to mean that a typical cytochrome oxidase is not present.

pH Studies

The enzyme systems studied appeared to be active over a wide range of pH with little alteration of the rate of O_2 uptake per minute from pH 5.8–7.4. A pH of 6.9–7.0 was selected for the experiments described and the extracts were maintained at this pH at all times.

Discussion

It has been well established that the terminal oxidase system of several different bacteria is exclusively associated with the particulate fraction (3, 7, 9, 11, 13). Furthermore, Alexander and Wilson (2) have presented evidence that, while the mechanism for the transport of electrons is solely a property of the granules, the interconversion of the tricarboxylic acids is a function of the non-sedimentable portion of the cell. On the other hand, Wilson and Wilson (14) demonstrated that the complete succinoxidase system remains in the supernatant even after centrifugation for 2–3 hours at 145,000 $\times$ *g*. Their evidence indicated that the succinoxidase of the bacterium survived in discrete units or particles which would have to be much smaller than mitochondria. There has also been considerable evidence from experiments with animal cells indicating that the electron-transport system may remain intact in submitochondrial units (8).

It has been generally agreed in the past that in animal cells the function of the soluble material is its role in catalyzing the anaerobic breakdown of glucose to lactic acid. Recently, however, Hochstein (6) has presented evidence that the mitochondria are enzymatic sites of intense glycolytic activity. The literature would seem to indicate that the site of glycolysis in bacteria is analogous to that in higher cells.

The evidence presented in the present work indicates that the localization of enzyme systems for the oxidation of glucose and gluconate is found to be present in both the particulate and non-sedimentable portions of the extract. The activity for the oxidation of gluconate seems to be evenly divided between the two fractions while activity for the oxidation of glucose seems to be approximately 40% in the non-sedimentable fraction and 60% in the particulate fraction. These conclusions are based on the observations: that the sum of the enzyme activities and quantity of protein found in the isolated fractions was equivalent to the amount found in the unfractionated extract; that the active fractions had a large percentage of the activities; and, that the ratio of the specific activities of the fractions to that of the extract was high. Furthermore, inhibitor studies with cyanide and the identification by chromatography of 2-ketogluconate as the end product of the reactions would indicate that the

same enzyme system occurred in each fraction. Also, since the same pattern of localization was observed regardless of the period of sonic oscillation, it is concluded that the sonic treatment did not "solubilize" the particles. At the same time, results obtained from extracts prepared by short-term alumina grinding, which is considered to be a gentler method of rupturing microbial cells, appeared to be identical. Therefore, the systems active for the non-phosphorylative oxidation of glucose and gluconate for this particular organism must exist in particles and also in either the non-particulate portion of the cell or in small particles that are non-sedimentable at the forces employed.

All of the activity for the oxidation of glucose-6-phosphate was consistently found to be located solely in the non-sedimentable fraction of the extract. The addition of the cofactors, ATP or DPN, or metal activators such as magnesium, manganese, zinc, iron, or calcium, did not stimulate oxidation of this substrate by the particulate fraction. At this time it is not entirely possible to say whether or not this enzyme system is located in the non-particulate portion of the cell, although the present evidence would so indicate.

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BIOSYNTHESIS OF COBALAMIN ANALOGUES BY PROPIONIBACTERIUM ARABINOSUM[1]

D. Perlman and J. M. Barrett

Abstract

Examination by filter paper partition chromatography and filter paper ionophoresis of the cobalamins* present in cultures of *Propionibacterium arabinosum* grown in a medium containing glucose, yeast extract, and salts showed that only adenine-hydroxo-cobalamin was present. Addition of 5,6-dimethylbenzimidazole to the growing cultures resulted in formation of a cobalamin containing this substance in the nucleotide portion of the molecule. Cobalamins containing benzimidazole, 5-methylbenzimidazole, 2,6-diaminopurine, benzotriazole, benzothiazole, and 5-nitrobenzimidazole in the nucleotide were prepared by this procedure and found to have the migration and biological characteristics of these cobalamins as previously described in the literature. New cobalamins have also been prepared presumably including those containing 5-trifluoromethylbenzimidazole, phenazine, 2-hydroxyphenazine, 4-bromo-6-methoxybenzimidazole, 3,4-dihydro-4-oxoquinazoline, 4-chloro-8-nitroquinazoline, and quinoxaline in the nucleotide. All of these new cobalamins were found to stimulate the growth of cobalamin-requiring cultures of *Escherichia coli*, *Lactobacillus leichmannii*, and *Ochromonas malhamensis*.

Introduction

Several years ago Leviton and Hargrove (11) described a microbial process for the production of vitamin B_{12} (5,6-dimethylbenzimidazole-hydroxo-cobalamin) based on the growth of *Propionibacterium freudenreichii* in nutrient media containing cobalt salts. More recently Pawelkiewicz and Nowakowsha (12) reported the synthesis of a number of cobalamin analogues as a result of adding substituted benzimidazoles to growing cultures of *P. shermannii*. Among the analogues produced were those in which the 5,6-dimethylbenzimidazole portion of the cobalamin nucleotide was replaced by one of the following: benzimidazole; 5-nitrobenzimidazole; 5-nitro-6-methylbenzimidazole; 5,6-dinitrobenzimidazole; benzotriazole; imidazole-benzimidazole; and 2-mercaptobenzimidazole. In this communication we wish to summarize some of our observations on the biosynthesis of these and some new cobalamin analogues by a culture of *P. arabinosum*.

Materials and Methods

Cultural Conditions

Stock cultures of *P. arabinosum* (ATCC 4965) were maintained in stabs of a tomato juice – peptone yeast extract agar and stored at 5° C. until needed. Inoculum was prepared by transferring viable cells to flasks of a medium containing yeast extract, glucose, and calcium carbonate, followed by incubation at 30° C. for 3 to 5 days. After two transfers in this medium a few milliliters of the cell suspension were used as inoculum for the cobalamin production.

[1]Manuscript received September 25, 1957.
Contribution from the Squibb Institute for Medical Research, New Brunswick, New Jersey, U.S.A.

*The nomenclature of Bernhauer and Friedrich (1) will be used in this communication. In this system vitamin B_{12} is named 5,6-dimethylbenzimidazole-cyano-cobalamin.

The fermentation medium contained 30 g. glucose, 20 g. autolyzed yeast, and 0.01 g. $Co(NO_3)_2.6H_2O$ and was diluted to 1 liter with tap water. Five hundred milliliter aliquots were placed in 1 liter Erlenmeyer flasks and the flasks were then plugged with non-absorbent cotton and autoclaved at 121° C. for 30 minutes. When the flasks had cooled to room temperature 10 g. of powdered calcium carbonate (sterilized by autoclaving followed by 3 hours' heating in an oven maintained at 150° C.) was added to each flask. A sterile solution of the substance to be tested as precursor for the cobalamin analogue was prepared by dissolving 50 mg. of the material in 10 ml. of 70% (v/v) aqueous ethanol and filtering the solution through a sterile ultrafine fritted glass filter (addition of a small amount of acid or alkali was sometimes necessary to dissolve the substance completely in the aqueous alcohol before filtration). One milliliter of this solution was added to the flask of sterile medium, and this was followed by addition of 5 to 10 ml. of the inoculum mentioned above. The inoculated flasks were placed on a reciprocating shaker (120 1-in. strokes per minute) located in a room maintained at 30° C. The *P. arabinosum* culture was allowed to grow in this medium for 6 days. At the end of each 24 hour period sufficient sterile 2 *N* potassium hydroxide was added to bring the pH of the growing culture to between pH 6.8 and 7.2. One milliliter of the alcoholic solution of the precursor was also added, so that at the end of the 6 day period approximately 60 mg. of the precursor had been added per liter of medium. After 3 days' incubation 30 ml. of a sterile glucose solution (50 g. glucose per 100 ml.) was also added to each flask. At the end of the 6 day period 50 ml. aliquots of the fermented medium were removed for analyses.

The substances used as precursors in these experiments were obtained with the cooperation of the Division of Medicinal Chemistry, Squibb Institute for Medical Research, and had been checked for purity and recrystallized before use if necessary.

Analytical Methods

The samples from the fermented media were prepared as follows before examination by filter paper chromatography and paper ionophoresis (followed by bioautography): Approximately 40 ml. of the sample (whose pH was about 6.5) was heated for 15 minutes in a boiling hot water bath. The suspension was then centrifuged and the collected solids were discarded. Approximately 0.5 ml. of an aqueous solution of potassium cyanide (5 g. per 100 ml.) was mixed with a 20 ml. aliquot of the clear supernatant liquid. An aliquot of this solution was assayed for cobalamin content by the bioassay method described in the *U.S. Pharmacopia* (15th edition) using the growth response of *Lactobacillus leichmannii* (ATCC 7830) with pure 5,6-dimethylbenzimidazole-cyano-cobalamin as reference standard. A second aliquot was analyzed by a modification of the bio-assay described by Ford and Porter (8), which uses the growth response of *Ochromonas malhamensis* and pure 5,6-dimethylbenzimidazole-cyano-cobalamin as reference standard. A third aliquot (approximately 10 ml. in volume) was thoroughly mixed with 10 ml. of a

phenol–benzene solution (70 parts 88% liquefied phenol, 30 parts benzene) and 3 g. ammonium sulphate on a mechanical shaker. The emulsion was separated by centrifugation and the non-aqueous top layer was transferred to a second test tube. Ten milliliters of *n*-butanol and 5 ml. of distilled water were added and the two phases were well mixed by vigorous shaking. The emulsion was broken by centrifugation, and the bottom aqueous layer containing the cobalamins present in the fermentation sample was removed for analysis and the upper layer was discarded.

Aliquots of the aqueous concentrate were analyzed by a paper ionophoretic method described by Holdsworth (9), using a 0.5 *N* acetic acid solution containing 0.02% potassium cyanide (w/v) as electrolyte and a voltage gradient of 8 v. per cm. for 18 hours across the strip of Whatman No. 3 MM paper. Aliquots of solutions of 5,6-dimethylbenzimidazole-cyano-cobalamin, adenine-cyano-cobalamin, 2-methyladenine-cyano-cobalamin, and Ford's factor B (7) were spotted on the paper strip (approximately 0.1 μg. of cobalamin per spot) parallel to the areas where the fermentation extracts were applied. The paper strips were dried after the 18 hour electrophoresis period, and then placed for 15 minutes on the surface of large agar plates which had been seeded with a cobalamin-requiring culture of *Escherichia coli* (ATCC 11105). (The agar medium contained 20 g. glucose, 1.2 g. citric acid, 0.4 g. $(NH_4)_2HPO_4$, 0.08 g. KCl, 0.42 g. $MgCl_2.4H_2O$, 0.04 g. $MnCl_2.4H_2O$, 0.023 g. $FeCl_3.6H_2O$, 0.02 g. $ZnCl_2$, 0.04 g. $CoCl_2.6H_2O$, 15 g. agar, 1 g. triphenyltetrazolium chloride, and sufficient distilled water to dilute the mixture to 1 liter. The triphenyltetrazolium chloride was sterilized separately and added to the liquefied agar medium just prior to pouring of the plates). After the 15 minute contact period the paper strips were removed and the agar plates were incubated at 37° C. for 18 hours. Zones of growth of the *E. coli* culture (visible as red zones due to the reduction of the tetrazolium dye to the colored formazan) were found on the agar plate in areas corresponding to the areas of the paper strip containing the cobalamins. In this ionophoretic system, 5,6-dimethylbenzimidazole-cyano-cobalamin was essentially neutral, while adenine-cyano-cobalamin, 2-methyladenine-cyano-cobalamin, and Ford's factor B moved approximately 4, 8, and 10 cm. from the point of application to the paper.

Other aliquots of the fermentation extracts were spotted on several sheets of Whatman No. 4 filter paper in parallel with spots of 5,6-dimethylbenzimidazole-cyano-cobalamin. Three solvent systems were used in developing the filter paper partition chromatograms. These included the *sec*-butanol – acetic acid (or NH_4OH) – water – 5%(w/v) KCN (100:1:50:0.25, by volume) system described by Ford *et al.* (7), and the *sec*-butanol – water – 5%(w/v) KCN (77:23:0.25, by volume) mixture saturated with $KClO_4$ used by Dellweg *et al.* (3). The chromatograms were developed at 35° C. for 18 hours and then dried at room temperature. The dried chromatograms were placed on the agar plates seeded with *E. coli* as described above. The plates were incubated at 37° C. for 18 hours and the positions of the zones of growth noted. Mobilities of the factors in the chromatograms were calculated relative to the mobility

of the 5,6-dimethylbenzimidazole-cyano-cobalamin standard. Under these conditions the 5,6-dimethylbenzimidazole-cyano-cobalamin moved about 27 cm. from the point of application in all three systems.

Experimental

Samples were removed from a *P. arabinosum* fermentation of the basal medium and analyzed by the methods mentioned above. The bio-assay using the growth response of *L. leichmannii* showed the presence of about 0.25 μg. per ml., 0.67 μg. per ml., and 0.79 μg. per ml. of cobalamin activity after 2, 4, and 6 days' incubation respectively. When *O. malhamensis* was used as the assay organism the cobalamin content was found to range from 0.003 to 0.004 μg. per ml. during the 6 day incubation period. Examination of the extracts by the filter paper chromatographic and paper ionophoretic systems described above showed that only adenine-cyano-cobalamin was present in these extracts. Similar data were obtained when the *P. arabinosum* culture was grown on a medium containing casein hydrolyzate, glucose, yeast extract, calcium carbonate, and cobalt nitrate, and a medium containing cornsteep liquor, glucose, calcium carbonate, and cobalt nitrate. When extracts taken from a fermentation of the autolyzed yeast – glucose – calcium carbonate – cobalt nitrate medium supplemented with 5,6-dimethylbenzimidazole were analyzed, the bio-assay using *L. leichmannii* was approximately the same as that obtained when the samples from the fermentation of the unsupplemented medium were analyzed. However, the assays based on the growth response of *O. malhamensis* were equal to those obtained with the *L. leichmannii* bio-assay. The paper chromatographic and paper ionophoretic analyses showed that the samples from the fermentation of the supplemented medium contained 5,6-dimethylbenzimidazole-cyano-cobalamin, and only a trace of adenine-cyano-cobalamin was present. Analyses of samples taken from fermentations supplemented with other benzimidazoles, purines, and related compounds showed that many of these substances were incorporated into the cobalamin nucleotide as a replacement for the adenine, as shown by the altered behavior of the cobalamin fraction in the paper ionophoretic and paper chromatographic systems. Some of the data obtained on the properties of these new cobalamins are summarized in Table I. The results obtained with the 2,6-diaminopurine-cyano-cobalamin, 5-methylbenzimidazole-cyano-cobalamin, benzotriazole-cyano-cobalamin, benzimidazole-cyano-cobalamin, and 5-nitrobenzimidazole-cyano-cobalamin agree with the data available in the literature.

The relationship of the compound added as precursor to the fermentation medium and the cobalamin produced is summarized in Table II. The culture of *P. arabinosum* was able to utilize many related compounds for the formula of a certain cobalamin. For example, benzimidazole-containing cobalamin was formed when either benzimidazole, dinitrobenzene, or *o*-phenylene diamine was added to the growing culture. The culture also formed some adenine-containing cobalamin in these fermentations, but the major portion of the cobalamin fraction was the benzimidazole-cobalamin. When other

TABLE I

BEHAVIOR OF COBALAMINS IN FILTER PAPER CHROMATOGRAPHY AND PAPER IONOPHORESIS

Cobalamin	Ionophoresis at pH 2.3 for 18 hr. at 8 v./cm.	Mobility in paper chromatography with reference to 5,6-dimethylbenzimidazole-cyano-cobalamin		
		System A	System B	System C
5-Methylbenzimidazole	Neutral	0.88	1.0	1.0
Benzimidazole	Neutral	0.90	0.95	0.95
Benzotriazole	Neutral	1.05	0.3	1.0
2,6-Diaminopurine	Equal to 2-methyladenine-cyanocobalamin	0.3	0.4	0.6
5-Nitrobenzimidazole	Neutral	0.85	1.0	1.0
Benzothiazole	Neutral	0.90	0.97	1.0
5-Trifluoromethylbenzimidazole	Neutral	0.95	0.95	0.95
4-Bromo-6-methoxy-benzimidazole	Neutral	0.74	0.78	1.0
3,4-Dihydro-4-oxo-quinazoline	Neutral	0.60	0.35	0.6
4-Chloro-8-nitro-quinazoline	Neutral	0.9	1.02	1.0
Phenazine	Neutral	0.5	0.6	0.35
2-Hydroxyphenazine	Neutral	0.9	0.8	0.9
Quinoxaline	Neutral	0.86	0.7	0.9
Adenine		0.25	0.3	0.6
5,6-Dimethylbenzimidazole	Neutral	1.0	1.0	1.0

NOTE: System A: $KClO_4$ – *sec*-butanol – 5%(w/v) KCN – water (100 mg.; 77 ml.; 0.25 ml.; 23 ml.).
System B: Acetic acid – *sec*-butanol – KCN – water (1 ml.; 100 ml.; 0.25 ml.; 50 ml.).
System C: NH_4OH – *sec*-butanol – KCN – water (1 ml.; 100 ml.; 0.25 ml.; 50 ml.).

TABLE II

BIOSYNTHESIS OF COBALAMINS BY *Propionibacterium arabinosum*

Cobalamin presumed formed	Precursor added to fermentation	Growth stimulating activity	
		L. leichmannii	*O. malhamensis*
5,6-Dimethylbenzimidazole	5,6-Dimethylbenzimidazole	+	+
	2,3-Dimethyl-5,6-diaminobenzene		
5-Methylbenzimidazole	5-Methylbenzimidazole	+	+
	3-Methyl-5,6-diaminobenzene		
Benzimidazole	Benzimidazole	+	+
	1,2-Dinitrobenzene		
	1,2-Diaminobenzene		
Benzotriazole	Benzotriazole	+	+
5-Trifluoromethylbenzimidazole	2-Nitro-4-trifluoromethylaniline	+	+
	4-Trifluoromethylphenylenediamine-1,2		
4-Bromo-6-methoxybenzimidazole	3-Bromo-5-methoxyphenylenediamine-1,2	+	+
2,6-Diaminopurine	2,6-Diaminopurine	+	–
5-Nitrobenzimidazole	5-Nitrobenzimidazole	+	+
Benzothiazole	Benzothiazole	+	+
3,4-Dihydro-4-oxo-quinazoline	4(3H)-Quinazolinone	+	+
4-Chloro-8-nitro-quinazoline	4-Chloro-8-nitro-quinazoline	+	+
Phenazine	Phenazine	+	+
2-Hydroxyphenazine	2-Hydroxyphenazine	+	+
Quinoxaline	Quinoxaline	+	+
Adenine	None	+	–

precursors were added including phenazines, quinazolines, substituted benzimidazoles, and quinoxaline, some adenine-containing cobalamin was formed, in addition to the new cobalamin. All of the new cobalamins stimulated the growth of the *L. leichmannii* culture, but to a different degree than the 5,6-dimethylbenzimidazole-cyano-cobalamin. Thus assay values in terms of the

latter are relatively meaningless since the dose–response curves were not parallel and most of the preparations contained some adenine-cyano-cobalamin. This was also true of the growth response of the *O. malhamensis* culture to stimulation of the new cobalamins, and for this reason the growth promoting activity is shown in Table II as positive or negative.

Discussion

The possibilities of biosynthesis of cobalamin analogues as a result of addition of precursors to growing cultures were first suggested by the experiments of Sahashi *et al.* (13) and Weygand *et al.* (14), who found that added 5,6-dimethylbenzimidazole was incorporated into the cobalamin formed by streptomycetes. More recently Ford *et al.* (7), Dellweg *et al.* (3, 4), and Bernhauer (2) reported that growing cells of cobalamin-requiring mutants of *E. coli* were able to synthesize cobalamin analogues if supplied with benzimidazoles or purines together with "factor B" (the portion of cobalamin exclusive of the 5,6-dimethylbenzimidazole containing nucleotide). Fantes and O'Callaghan (5, 6) have reported that addition of a number of benzimidazoles or related compounds to growing cultures of *Streptomyces griseus*, *S. olivaceus*, *S. fradiae*, *S. aureofaciens*, *Bacillus megaterium*, *Clostridium welchii*, and *C. tetani* resulted in formation of new cobalamin analogues containing the added substances in the cobalamin nucleotide. They concluded that only certain cultures would utilize some of the precursors, and it is apparent from their studies and those of Pawelkiewicz and Nowakowska (12) mentioned above that bacterial cultures have less specific requirements for the substances accepted as precursors.

In the experiments summarized in Table II we found that a *P. arabinosum* is also able to synthesize these cobalamin analogues previously reported as products of other biosynthetic methods. These include cobalamins containing the following bases in the nucleotide portion of the molecule: 5-methylbenzimidazole; benzimidazole; benzotriazole; benzothiazole; 2,6-diaminopurine; and 5,6-dimethylbenzimidazole. When no precursor is added to the *P. arabinosum* culture the cobalamin produced contained adenine in the nucleotide portion of the molecule. In some of the experiments summarized in Table II a number of precursors not previously mentioned in the literature were tested and were incorporated into the cobalamin formed by the *P. arabinosum* culture as shown by filter paper chromatographic and filter paper ionophoretic methods. On the basis of this evidence it is presumed that the new cobalamins contained the following bases in the nucleotide portion of the cobalamin molecule: 5-trifluoromethylbenzimidazole; 4-bromo-6-methoxybenzimidazole; 3,4-dihydro-4-oxo-quinazoline; 4-chloro-8-nitro-quinazoline; phenazine; 2-hydroxyphenazine; and quinoxaline.

All of the new cobalamins formed by the biosynthesis process using *P. arabinosum* stimulated the growth of cobalamin-requiring cultures of *E. coli*, *L. leichmannii*, and *O. malhamensis* commonly used in the bio-assay for cobalamin content of natural materials. These studies indicate that the active

compounds contain a benzimidazole, a phenazine, a quinoxaline, a quinazoline, a benzotriazole, or a benzothiazole in the nucleotide portion of the molecule. Replacements of the benzene ring with a purine results in loss of activity, while introduction of halide, hydroxyl, or nitro groups does not affect ability to stimulate the growth of the *O. malhamensis* culture. These relationships are of interest in view of Kon's suggestion (10) that cobalamins stimulating the growth of *O. malhamensis* will stimulate the growth of chicks fed a ration deficient in cobalamins.

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DÉTECTION MICROSCOPIQUE DE LA FLUORESCENCE PRIMAIRE CHEZ LES BACTÉRIES[1]

J. DE REPENTIGNY ET S. SONEA

Abstract

Thirty-one bacterial species showed a yellow primary fluorescence (autofluorescence) which could be observed visually with a commercial fluorescence microscope and recorded on photographic emulsions.

The present work emphasizes the importance of this natural property for the study of bacteria, either for taxonomic purposes or as a preliminary step when using fluorochromes. The lack of an easy and reliable technique for detecting the existence of primary fluorescence in bacteria is probably the reason why very little work has been done in this field. Nothing is quoted about this property of bacteria in most textbooks of bacteriology.

We deliberately left over the apparently related subject of the well-known macroscopic fluorescence of some *Pseudomonas*, *Azotomonas*, and *Azotobacter* cultures which is associated with diffusible pigments equally present inside and outside the bacteria. The aim of our study was the detection of the primary fluorescence of the bacterial cell itself.

In order to examine and photograph the primary fluorescence of bacteria with greater accuracy, two important improvements in technique were introduced (*a*) elimination of fluorescent particles, present in all the culture media which we used, by the simple expedient of growing the bacteria on cellophane, (*b*) increased reliability of black and white microphotographs through the use of the bright-field condenser. By using the old and usual method of fluorescence microscopy with the dark-field condenser and ultraviolet rays, it is not always possible to determine on black and white photomicrographs whether or not a given bacterium is fluorescent. With the latter setup the images can be produced not only by the visible light emitted by the fluorescent bacteria but also by the unfiltered rays which eventually pass through the system of filters during long exposures. To overcome this difficulty, we now use the bright-field condenser with ultraviolet or blue rays and the appropriate selective filters. The specimens are best prepared as dry smears, covered with an Euphos selective filter (cover glass) using no immersion liquid. This combination is more likely to give reliable information on the presence or absence of fluorescence.

Introduction

Plusieurs auteurs ont récemment publié sur la microscopie à fluorescence, mais le seul objet de leurs études fut la *fluorescence secondaire* des bactéries. Ils ont employé surtout les deux méthodes suivantes:

1. Coloration directe des bactéries vivantes ou mortes par des fluorochromes (acridine orange-NO, rhodamine-B, auramine-O, etc.). L'application de cette technique à la recherche du bacille tuberculeux dans les produits pathologiques est bien connue (6, 15 et 18).

2. L'utilisation d'anticorps couplés à la fluorescéine (4), permettant la détection (7, 8, 12 et 14) et la localisation spécifique des antigènes dans les microorganismes (7, 8 et 16).

[1]Manuscrit reçu le 7 octobre 1957.
Contribution de l'Institut de Microbiologie et d'Hygiène et du Département de Bactériologie de la Faculté de Médecine de l'Université de Montréal. Cette recherche a été partiellement subventionnée par le Ministère de la Santé de la Province de Québec (subvention fédérale–provinciale à la recherche sur la Santé Publique) et par un octroi des Fondations Joseph Rhéaume.

Notre présent travail porte sur une autre application de la microscopie à fluorescence, trop négligée dans le passé. Il s'agit de la *fluorescence primaire*, c'est-à-dire celle qui est émise par les substances bactériennes elles-mêmes, sans l'aide de colorants étrangers aux bactéries.

Le phénomène de la fluorescence primaire a été depuis longtemps signalé chez les cellules végétables et animales, mais il est passé presque inaperçu chez les bactéries, à quelques exceptions près. En 1917 et 1921, Kaiserling (11) a révélé l'existence de la fluorescence primaire chez 14 espèces bactériennes, dont le bacille tuberculeux. En autant que nous le sachions, ces constatations n'ont pas été confirmées et furent oubliées par la suite. Depuis, seules les bactéries suivantes ont été mentionnées dans la littérature comme présentant cette propriété: *Bacillus anthracis*, *B. megaterium* et *B. mycoides* (2) et *Pseudomonas aeruginosa* (12). Pour éviter toute confusion, il faut mentionner que les groupes de bactéries dites "fluorescens" (qui sont d'ailleurs en grande partie des *Pseudomonas* ou *Azotomonas*) et les *Azotobacter* (9, 10) ne présentent pas d'intérêt particulier dans notre étude sur la détection microscopique de la fluorescence primaire de la cellule bactérienne. En effet, dans le cas de ces espèces, il s'agit d'une fluorescence observée macroscopiquement, et qui est due à des pigments qui diffusent autour des bactéries.

Nous avons eu l'occasion de remarquer que l'intensité de la fluorescence primaire des staphylocoques pouvait égaler l'intensité de ces mêmes bactéries traitées par des anticorps fluorescents. Ce fait nous a poussés à entreprendre une étude de la fluorescence primaire des staphylocoques et de quelques autres espèces.

Au cours de ce travail, nous avons été obligés de modifier les techniques d'examen microscopique par fluorescence afin de pouvoir mieux interpréter les images observées.

Matériaux et Techniques

Souches bactériennes

Nous avons utilisé les souches de staphylocoques Wood 46 et A8, ainsi que les souches 728, 740, 746, 785, 787 et 789, ces dernières récemment isolées dans nos laboratoires et provenant d'infections humaines. Parallèlement, nous avons examiné une souche de chacune des trente autres espèces énumérées dans le tableau I. Toutes ces souches ont été cultivées pendant 24 heures, sur les milieux appropriés.

FIG. 1. Staphylocoques lavés, rayons bleus, condensateur à fond clair et filtres appropriés. Exposition 2 minutes.

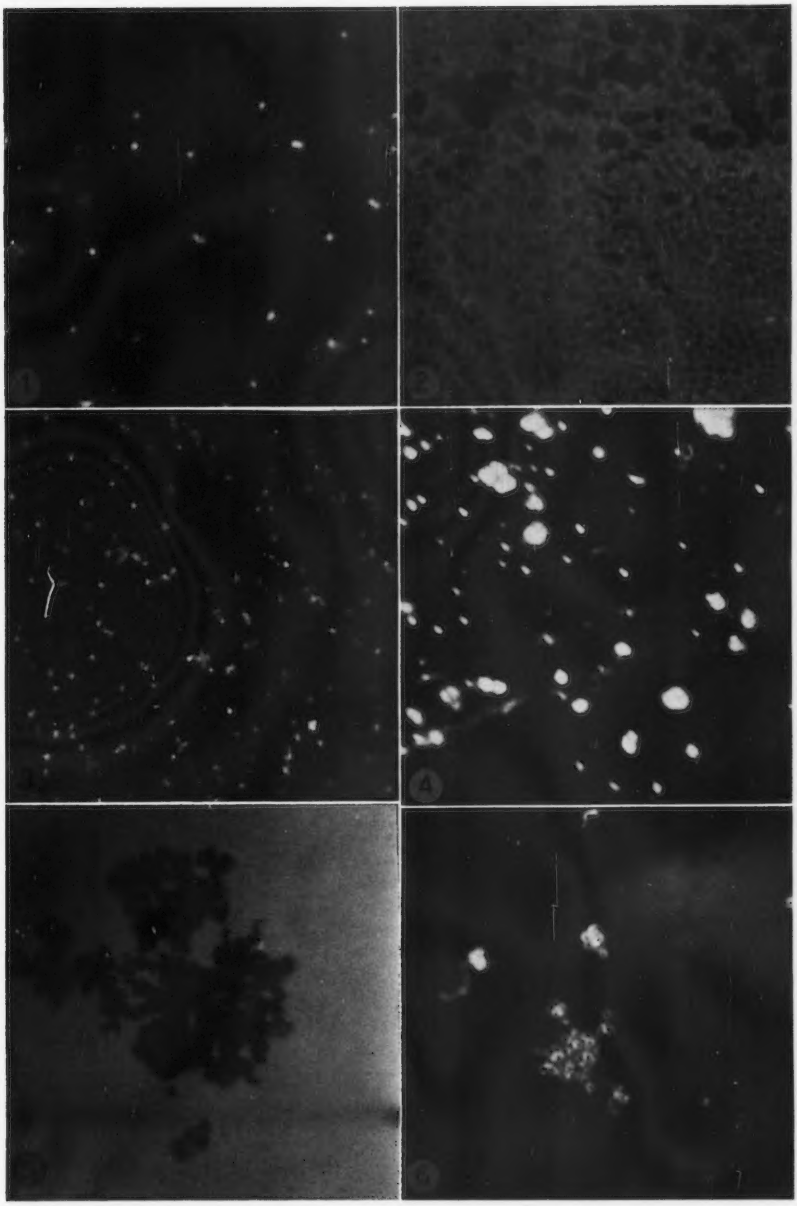
FIG. 2. Staphylocoques lavés, rayons ultraviolets, condensateur à fond clair et filtre OG1, 2.5 mm. dans l'oculaire et lamelle Euphos. Exposition 8 minutes.

FIG. 3. *Gaffkya tetragena*, rayons ultraviolets, condensateur à fond clair, et filtres comme en Fig. 2. Exposition 32 minutes.

FIG. 4. Particules de charbon, non fluorescentes à notre examen visuel, rayons ultraviolets condensateur à fond noir, filtres appropriés. Exposition 32 minutes.

FIG. 5. Particules de charbon, non fluorescentes à notre examen visuel, rayons bleus, condensateur à fond clair, filtres appropriés. Exposition 32 minutes.

FIG. 6. Staphylocoques lavés, rayons ultraviolets condensateur à fond noir, filtres appropriés. Exposition 4 minutes.



Examen visuel et microphotographie

Ces examens ont été effectués avec un microscope à fluorescence commercial fabriqué par la maison Leitz, modèle BX, muni de ses accessoires de microphotographie. Les microphotographies ont été prises sur des films Kodak Plus X, 35 mm. et ceux-ci ont été développés à 24° C., pendant 8 minutes, dans le Dektol Kodak.

Améliorations apportées à l'examen des bactéries par la microscopie à fluorescence pour détecter avec certitude la fluorescence primaire

1. Milieux de culture

Les milieux de culture empiriques contiennent des substances fluorescentes en solution ou en suspension et la présence de ces dernières dans le frottis des bactéries prête à confusion. Selon nos observations, même le lavage ne peut débarrasser les bactéries des fragments microscopiques fluorescents qui proviennent des milieux de culture utilisés. Nous avons confirmé ce fait par l'examen microscopique des sédiments obtenus en centrifugeant les milieux stériles et par conséquent exempts de bactéries. Pour éliminer ces particules qui constituaient une cause d'erreur possible, nous avons utilisé comme spécimens dans nos derniers examens microscopiques des bactéries cultivées sur un film de cellophane déposé à la surface des milieux gélosés. Des colonies d'apparence normale se développent sur ces films de cellophane. Les frottis effectués à partir de ces colonies montrent presque exclusivement des bactéries et seulement un ou deux débris fluorescents par champ microscopique.

2. Préparation et examen des frottis sur lames

Nous avons d'abord examiné les frottis fixés par la chaleur, avec un objectif à immersion. Aprés avoir déposé sur le frottis une goutte de glycerol, on recouvrait ce dernier d'une lamelle "Euphos" qui arrête la plus grande partie des rayons ultraviolets et bleus. Nous avons noté que le liquide d'immersion pouvait facilement dissoudre des substances fluorescentes d'origine bactérienne, ce qui ne permettait pas d'obtenir un champ microscopique suffisamment sombre. Nous avons contourné cette difficulté en recouvrant d'une lamelle "Euphos" le frottis non fixé et sec, et en utilisant pour l'examen un objectif sec 45X. Dans ces conditions, les bactéries qui sont fluorescentes se détachent nettement sur le fond très sombre (Fig. 1, 2 et 3).

3. Choix du condensateur

La vieille méthode de l'examen microscopique avec condensateur à fond noir et les rayons ultraviolets a été utilisée récemment et d'une façon exclusive par de nombreux chercheurs (3, 5, 8, 14, 16, 17, 19 et 20). Pourtant, avec cette méthode, le microscope et les filtres peuvent être traversés par quelques rayons de lumière (1) et, dans certains cas, un objet microscopique considéré comme non fluorescent peut impressionner le film photographique (voir Fig. 4). Si l'on allonge le temps d'exposition, l'image prend dans ce cas l'aspect familier d'une microphotographie sur fond noir. On peut facilement prendre cet aspect pour une preuve photographique de fluorescence. La constatation

d'une fluorescence a beaucoup plus de valeur quand on la fait en regardant au microscope. Pour avoir une preuve photographique de fluorescence sur film noir et blanc, avec rayons ultraviolets ou bleus, il vaut mieux employer le condensateur à fond clair, avec les filtres Euphos sur la lame et OG1 dans l'oculaire. Ce système, en arrêtant les rayons de courte longueur d'onde, réalise un champ microscopique foncé, sur lequel les bactéries fluorescentes se détachent nettement à cause de leur luminosité. Dans ces conditions, on ne peut obtenir de fausses images de fluorescence qui seraient dues exclusivement aux rayons parasites qui traversent en faible quantité le système optique. En effet, les microphotographies surexposées de particules non fluorescentes montrent des images contraires, soit un champ microscopique clair et des particules foncées (Fig. 5). Dans ces conditions, l'image photographique peut servir comme preuve incontestable de l'absence ou de la présence d'une fluorescence. En tenant compte de ces constatations, nous avons essayé de tirer profit et du condensateur à fond clair et du condensateur à fond noir, avec lumière bleue ou ultraviolette.

Résultats

Nous avions donc établi une technique sûre avant de déterminer l'existence ou l'absence de la fluorescence primaire chez certaines bactéries. Il découle de ces travaux préliminaires que la méthode de choix doit comporter les modifications suivantes (telles que décrites plus haut dans la section "Améliorations apportées à l'examen des bactéries par la microscopie à fluorescence pour détecter avec certitude de la fluorescence primaire"):

1. les bactéries sont cultivées sur film de cellophane déposé à la surface des milieux de culture gélosés;
2. les frottis sont séchés à l'air sans fixation par la chaleur et sont recouverts d'une lamelle Euphos;
3. l'emploi d'un objectif sec 45× et d'un oculaire 8×, muni d'un filtre OG1 et du condensateur à fond clair.

Au moyen de cette méthode, nous avons constaté l'existence d'une fluorescence primaire chez les trente et une espèces examinées, dont huit souches de staphylocoques (Tableau I). Ces observations ont pu être répétées à volonté. La fluorescence primaire des staphylocoques et de la plupart des espèces examinées était bien visible à l'œil quand on regardait dans le microscope. Avec le condensateur à fond noir et les rayons ultraviolets, les cocci examinés se présentent comme des anneaux lumineux, leur périphérie seule paraissant fluorescente (Fig. 6). Par contre, en utilisant le condensateur à fond clair avec la lumière bleue ou ultraviolette, les filtres OG1 dans l'oculaire et Euphos sur la lame, les cocci apparaissent comme des points jaunes lumineux mais sans éclat. Toute la bactérie semble également fluorescente et l'aspect d'anneau n'existe plus (Fig. 1, 2 et 3). L'utilisation du fond clair donne donc une image plus vraie et plus facilement interprétable.

Les images vues en regardant au microscope peuvent être photographiées en noir et blanc en utilisant l'appareil décrit plus haut. Ces microphotographies représentent fidèlement les observations visuelles à l'exception de

celles qui ont été surexposées avec le condensateur à fond noir. On peut apprécier l'intensité de la fluorescence par le temps minimum d'exposition photographique qui a varié de 2 à 32 minutes, selon les diverses techniques et souches utilisées. En effet, quelques frottis ont montré une fluorescence plus intense que les autres; cependant, il nous semble difficile de préciser les différences d'intensité de la fluorescence primaire, d'un examen à l'autre, dans nos conditions expérimentales. Nous essayons dans le tableau I de représenter les différences d'intensité telles qu'elles ont pu être constatées en regardant au microscope.

TABLEAU I

APPRÉCIATION VISUELLE AU MICROSCOPE DE LA FLUORESCENCE PRIMAIRE DE DIVERSES ESPÈCES BACTÉRIENNES

Intensité faible (1)	Intensité moyenne (II)	Intensité importante (III)
Corynebacterium hoffmanii	*Escherichia coli* 055	*Bacillus megaterium*
Aerobacter aerogenes	*Escherichia coli* 0111	*Bacillus subtilis*
Escherichia coli var. *communior*	*Klebsiella pneumoniae*	*Corynebacterium diphtheriae*
Proteus mirabilis	*Shigella dysenteriae*	*Staphylococcus aureus**
Proteus vulgaris	*Shigella sonnei*	*Streptococcus pyogenes*
Salmonella aertrycke	*Shigella flexneri*	*Gaffkya tetragena*
Salmonella gallinarum	*Paracolobactrum aerogenoides*	*Sarcina lutea*
Serratia marcescens	*Salmonella hirschfeldii*	
Pseudomonas aeruginosa	*Mycobacterium phlei*	
Mycobacterium ranae	*Streptococcus agalactiae*	
Mycobacterium smegmatis	*Streptococcus faecalis*	
Mycobacterium tuberculosis	*Propionibacterium shermanii*	

NOTE:
(I) L'image observée est celle des masses bactériennes; les bactéries ne peuvent être vues individuellement.
(II) On peut observer la fluorescence de chaque cellule bactérienne.
(III) Même caractéristique (II), mais avec une intensité plus forte.
*Toutes les huit souches de staphylocoques examinées ont montré une fluorescence d'une intensité pratiquement égale.

Discussion

Du point de vue technique, nous avons rencontré plusieurs difficultés au cours de notre présent travail. Nous avons indiqué au chapitre "Matériaux et techniques" les solutions que nous avons apportées à ces dernières. Une partie de ces difficultés est due à la relativité d'un grand nombre de propriétés physiques du matériel employé. Le système de filtres n'élimine jamais tous les rayons parasites (1); il est difficile d'autre part de trouver des substances organiques dépourvues de toute fluorescence primaire, etc. Nous tenons à souligner le fait que les différences sont toujours quantitatives et non pas absolues.

La fluorescence primaire est une propriété courante des bactéries, ainsi que l'indiquent nos résultats. Ceux-ci montrent l'importance de cette propriété, pour les études bactériologiques. Les caractéristiques de la fluorescence primaire de chaque espèce pourraient s'ajouter aux critères sur

lesquels se basent la classification et la description des bactéries. On devrait rechercher des précisions supplémentaires sur le choix de la longueur d'onde de la source lumineuse et sur l'intensité et la couleur (10) de la fluorescence des différentes espèces bactériennes. Comme exemple, citons le cas du bacille diphtérique qui montre une fluorescence primaire beaucoup plus forte que celle du pseudo-diphtérique (Tableau I). Dans nos conditions expérimentales, *Klebsiella pneumoniae* possède une fluorescence plus forte qu'-*Aerobacter aerogenes* (cf. Tableau I). Nos résultats ont montré que l'intensité de la fluorescence des staphylocoques ne variait pas sensiblement d'une souche à l'autre. Le cas des autres espèces est à l'étude.

Il n'est pas fait mention dans les traités actuels de bactériologie, de la fluorescence primaire des bactéries. Le travail avant-coureur de Kaiserling (11) n'a pas attiré l'attention des bactériologistes durant les 36 années qui ont suivi sa publication. Ceci explique pourquoi Levaditi (13) a redécouvert la fluorescence de *Pseudomonas aeruginosa*. Nous avons nous-mêmes complété le présent travail avant de découvrir cette publication déjà ancienne. Nous avons donc confirmé l'existence de la fluorescence primaire chez plusieurs espèces bactériennes. Cependant, nous n'avons pas vu les diverses couleurs décrites par Kaiserling. Il est difficile d'apprécier les résultats de cet auteur, qui ne sont pas accompagnés d'images photographiques et qui ont été obtenus avec un microscope à fluorescence très exclusif.

Nous sommes arrivés à cette conclusion que la fluorescence primaire de toute bactérie devrait être vérifiée avant d'entreprendre une étude nouvelle sur la fluorescence secondaire, à l'aide de fluorochromes. Dans le cas du bacille tuberculeux, l'intensité de la première étant faible, elle peut difficilement être confondue avec la forte intensité d'une bactérie colorée à l'auramine-O, par exemple. Très différent est le cas des staphylocoques: Le seuil supérieur de leur fluorescence primaire est si élevé qu'il a pu égaler, dans nos expériences, le seuil inférieur de la fluorescence secondaire conférée à ces germes lorsque traités par des anticorps couplés à la fluorescéine. Comment distinguer dans ces conditions la fluorescence secondaire de la fluorescence primaire? Ni l'œil au microscope, ni la photographie en noir et blanc ne sont capables de distinguer l'une de l'autre. Il est entendu qu'on pourrait trouver des techniques appropriées de travail, par des jeux de filtres différents, par l'emploi de films sélectifs en couleurs et surtout par le couplage aux anticorps de fluorochromes donnant des couleurs bien différentes de celle de la fluorescence primaire (17). Une étude semblable nous semble difficile à réaliser dans le cas des staphylocoques, lorsqu'on utilise un microscope à fluorescence standard et des anticorps couplés à la fluorescéine.

Résumé et Conclusion

1. Nous avons mis au point des méthodes de préparation des cultures et d'examen au microscope à fluorescence qui permettent de déceler sans erreur l'existence de la fluorescence primaire chez un grand nombre d'espèces bactériennes.

2. Les trente et une espèces bactériennes examinées ont présenté une fluorescence primaire non négligeable. Nous avons classé approximativement les espèces examinées en trois groupes d'après l'intensité de leur fluorescence, telle que vue au microscope.

3. Les caractéristiques de la fluorescence primaire des bactéries pourraient servir à leur description et à leur classification.

4. Comme la fluorescence primaire nous semble très répandue, l'appréciation de la fluorescence secondaire, apparaissant sous l'effet de colorants fluorescents, ne saurait être basée que sur des différences quantitatives.

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HEXOSE PHOSPHATE METABOLISM BY ACETOBACTER MELANOGENUM[1]

H. KATZNELSON

Abstract

Cell-free preparations of *Acetobacter melanogenum* readily oxidized glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, 6-phosphogluconate, and ribose-5-phosphate; fructose-1,6-diphosphate was utilized very slowly. The presence of an active triphosphopyridine nucleotide (TPN)-linked glucose-6-phosphate dehydrogenase and of phosphohexose isomerase was demonstrated; phosphoglucomutase was also present in these extracts. 6-Phosphogluconate dehydrogenase activity (TPN-linked) was low in extracts of glucose-grown cells, but was high in sonates of gluconate-grown cells. An active oxidizing system for reduced diphosphopyridine nucleotide was present but transhydrogenase was not detected. Pyruvate was produced extensively from 6-phosphogluconate but only slowly from ribose-5-phosphate; fluoride inhibited pyruvate formation from both substances. Ribose-5-phosphate was degraded readily via the transketolase-transaldolase series of reactions. Glyceraldehyde-3-phosphate dehydrogenase, hexokinase, gluconokinase, 2-ketogluconokinase, and aldolase were detected but not phosphohexokinase. The results suggest that glucose is oxidized either directly or after phosphorylation but is not metabolized glycolytically. The oxidation of hexose phosphate appears to occur predominantly as a result of the splitting of 6-phosphogluconate to pyruvate and glyceraldehyde-3-phosphate.

Introduction

In previous papers (3, 13) it was reported that cell-free preparations of *Acetobacter melanogenum* oxidized glucose, gluconate, and 2-ketogluconate to 2,5-diketogluconate, an observation which was confirmed recently by Kondo *et al.* (15). During the past year it was found (4) that in the presence of phenazine methosulphate 2,5-diketogluconate was oxidized further, one of the products being α-ketoglutarate. It has been found in addition (14), that glucose-1-phosphate (G-1-P) and glucose-6-phosphate (G-6-P) also were oxidized by these preparations. This indicates the existence in *A. melanogenum* of an alternate system for glucose oxidation as has been demonstrated also in *Pseudomonas fluorescens* (20, 21), *Corynebacterium creatinovorans* (8), and other bacteria (2, 16, 19). More recently Hauge *et al.*(10) reported on the oxidation of phosphorylated carbohydrates via the pentose cycle by *Acetobacter suboxydans*. The results presented herein concern hexose phosphate metabolism by *A. melanogenum*.

Materials and Methods

Cells of *A. melanogenum* (MA62) were grown in a medium containing 1% yeast extract, 0.5% tryptone, 0.1% K_2HPO_4, 2% dextrose, and 0.25% $CaCO_3$ which was sterilized separately and added aseptically. After incubation for 24 hours at 28° C. on a rotary shaker the cells were removed and washed twice in distilled water. Extracts were first prepared by grinding the cells with

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twice their weight of alumina powder with the gradual addition of cold $M/100$ phosphate buffer at pH 6.0. The alumina and cell debris were then removed by centrifugation in the cold for 5 minutes at 10,000 r.p.m. For the majority of the experiments reported herein, however, the cells were suspended in five times their wet weight of distilled water and disrupted by sonic vibration in a Raytheon 10 kc. sonic oscillator for 10 minutes. The sonic extract was centrifuged and the supernatant fluid containing approximately 22 mg. of protein per ml. used. The reagents, substrates, and methods used were those commonly employed at the Laboratory of Bacteriology, Department of Dairy Science, University of Illinois, Urbana, Illinois, where the major portion of this work was done, and have been described by Wood and Schwerdt and others (17, 19, 20, 21). The author is deeply grateful to Dr. W. A. Wood for his interest in this work and for his many valuable suggestions. The assistance of Dr. M. Kritchewsky of that laboratory is also gratefully acknowledged.

Results

Hexose Phosphate Metabolism

The results of an experiment with an extract of alumina-ground cells are shown in Fig. 1. It is clear that hexose phosphates were readily oxidized with one exception, fructose-1,6-diphosphate (F-1,6-P) which was utilized very slowly. 6-Phosphogluconate (6-PG) and ribose-5-phosphate (R-5-P) also were oxidized.

The work reported in all the following experiments was conducted with sonic extracts. The presence in these of an active triphosphopyridine nucleotide (TPN)-linked G-6-P dehydrogenase and phosphohexose isomerase may be noted in Table I. A somewhat less active phosphoglucomutase was demonstrated by the spectrophotometric method. The activity of 6-PG dehydrogenase was surprisingly low in these preparations. Wood and Schwerdt (21) also noted considerably lower activity of this enzyme derived from *P. fluorescens* than of G-6-P dehydrogenase. However, when cells of *A. melanogenum* were grown in a gluconate – yeast extract medium a very active TPN-linked 6-PG dehydrogenase was found. Diphosphopyridine nucleotide (DPN) was not reduced in the presence of G-6-P or 6-PG, under these conditions.

In an attempt to demonstrate TPNH (reduced TPN) oxidase activity, spectrophotometric tests were made in the presence of excess TPN and limiting G-6-P (Fig. 2). When TPN reduction was over, the contents of the cuvette were stirred vigorously but little reoxidation was observed. When DPN was added to this system no change in optical density occurred. However, when DPNH (reduced DPN) was added a sharp increase in optical density took place followed by an equally rapid decrease. These results suggested the presence of an active DPNH oxidizing system but little if any transhydrogenase. If the latter had been present there should have been a decrease in optical density on addition of DPN. The presence of an active DPNH oxidase may account for the observation that DPN was not reduced in the presence of G-6-P or 6-PG although it is quite possible that the corresponding

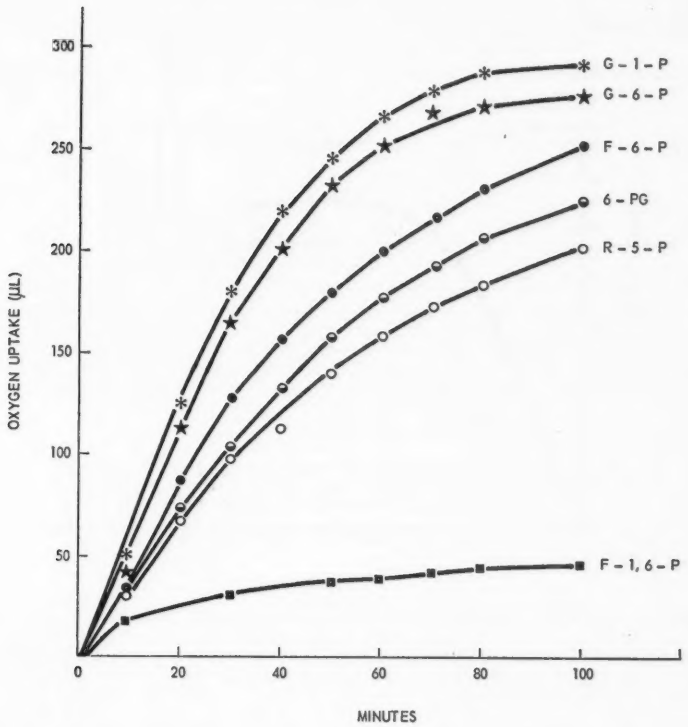

FIG. 1. Oxidation of hexose and pentose phosphates by a cell-free extract of aluminum-ground cells of *Acetobacter melanogenum*. The Warburg vessels contained: 0.5 ml. cell-free extract; 1.5 ml. *M*/100 phosphate buffer, pH 6.0; 0.1 ml. *M*/20 substrate; 0.2 ml. 20% KOH in center well. 30° C.

TABLE I

REDUCTION OF TRIPHOSPHOPYRIDINE NUCLEOTIDE (TPN) BY A SONIC EXTRACT OF *Acetobacter melanogenum**

Substrates	Q_{OD}†
Glucose-6-phosphate	20.0
Fructose-6-phosphate	10.0
Glucose-1-phosphate	3.3
6-Phosphogluconate	1.4

*The reaction mixture in the cuvette contained: 0.4 ml. glycylglycine buffer (*M*/4, pH 7.4); 0.3 ml. $MgCl_2$ (*M*/10); 0.1 ml. TPN (1.4 μM./ml. pH 7.0); sonic extract 0.01 to 0.1 ml.; 0.1 ml. substrate (*M*/10); water to 3.0 ml.

†Q_{OD} = Change in optical density at 340 mμ per mg. protein per hour.

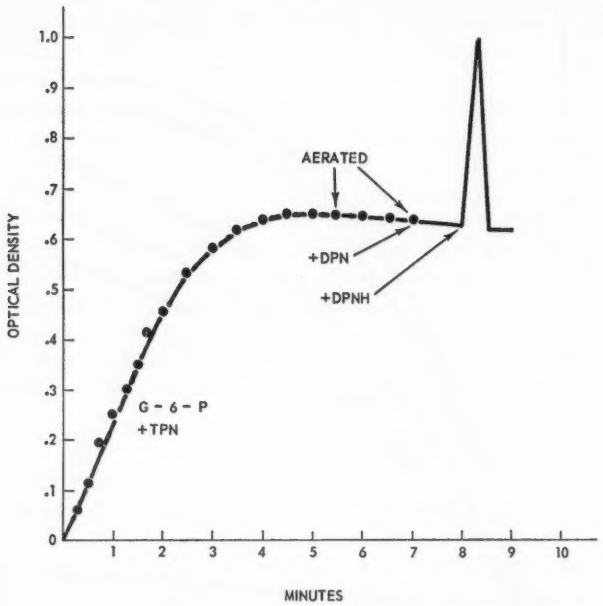

FIG. 2. Oxidation of reduced diphosphopyridine nucleotide (DPNH) by a sonic extract of *A. melanogenum*. The reaction mixture in the cuvette contained: 0.4 ml. glycylglycine buffer (*M*/4, pH 7.4); 0.3 ml. $MgCl_2$ (*M*/10); 0.5 ml. TPN (1.4 μM./ml., pH 7.0); 0.03 ml. sonic extract; 0.25 μM. glucose-6-phosphate; water to 3.0 ml.; 0.14 μM. of DPN or DPNH were added as indicated.

dehydrogenases are TPN-specific. Further confirmation of the presence of a DPNH oxidase was obtained by adding DPNH to a cuvette containing all the necessary ingredients except enzyme and substrate. A rapid increase in optical density resulted; the addition of sonic extract caused a very rapid decrease in optical density.

The degradation of 6-PG was followed by measuring pyruvate production in the presence of arsenite or fluoride, and pentose formation. As may be seen in Table II, approximately 6 μM. pyruvate were produced from 6.7 μM. 6-PG; this yield is close to the theoretical figure assuming a split of 6-PG to glyceraldehyde-3-phosphate (G-3-P) via the Entner–Doudoroff pathway (5). Only a small amount of pentose was detected in these experiments even after addition of TPN. Essentially similar results were obtained with gluconate-grown cells, as shown in Table III which also includes data on pyruvate production from R-5-P and from 6-PG+TPN. It may be noted that a considerably greater amount of pyruvate was obtained from 6-PG alone or with arsenite than from R-5-P. Fluoride inhibited pyruvate formation from both substrates. TPN was added to 6-PG in order to facilitate its conversion to pentose; however, under these conditions almost twice as much pyruvate was produced (in 20 minutes) as from 6-PG alone; this reaction was also

TABLE II

PRODUCTION OF PYRUVATE FROM 6-PHOSPHOGLUCONATE (6-PG) BY A SONIC EXTRACT OF GLUCOSE-GROWN CELLS OF *Acetobacter melanogenum**

Substrate and inhibitors	Pyruvate (μM.)
None	1.1
Fluoride	1.0
Arsenite	1.2
6-Phosphogluconate	6.0
6-Phosphogluconate + arsenite	5.8
6-Phosphogluconate + fluoride	1.0

*The reaction mixture contained: 0.2 ml. sonic extract; 0.2 ml. tris (hydroxymethylaminomethane) buffer (M/1, pH 7.65); 0.1 ml. reduced glutathione (10 mg./ml.); 0.1 ml. $FeSO_4.7H_2O$ (33 mg./2 ml.); 0.1 ml. arsenite (M/50, pH 7.8); 0.15 ml. fluoride (M/2); 0.05 ml. 6-PG (135 μM./ml.) and water to 1.0 ml. Incubation, 45 minutes at 37° C. Reaction stopped by addition of 1 ml. of an 0.1% solution of 2,4-dinitrophenylhydrazine in 2 N HCl.

TABLE III

PYRUVATE PRODUCTION FROM RIBOSE-5-PHOSPHATE (R-5-P) AND 6-PHOSPHOGLUCONATE (6-PG) BY EXTRACTS OF GLUCONATE-GROWN CELLS OF *Acetobacter melanogenum**

Substrates	Pyruvate (μM.)		
	0	20 min.	40 min.
Ribose-5-phosphate	0.18	0.78	1.1
Ribose-5-phosphate + arsenite	0.14	1.4	1.9
Ribose-5-phosphate + fluoride	0.24	0.25	—
6-Phosphogluconate	0.13	3.0	4.7
6-Phosphogluconate + arsenite	0.12	3.4	5.8
6-Phosphogluconate + fluoride	0.12	0.14	0.13
6-Phosphogluconate + triphosphopyridine nucleotide (TPN)	0.18	5.76	—
6-Phosphogluconate + TPN + fluoride	0.14	0.18	—

*The reaction mixture contained: 0.5 ml. sonic extract; 0.4 ml. tris (hydroxymethylaminomethane) buffer (M/1, pH 7.5); 0.2 ml. reduced glutathione (10 mg./ml.); 0.2 ml. $FeSO_4.7H_2O$ (33 mg. per 2 ml.); 0.3 ml. arsenite (M/20, pH 7.8); 0.3 ml. fluoride (M/2); 0.1 ml. substrate-6-PG or R-5-P (M/10); 0.3 ml. TPN (0.003 M); water to 3 ml. Pyruvate was determined on 0.5 ml. aliquots and the results recalculated on a 3 ml. basis.

fluoride sensitive. Since comparatively little pyruvate was formed from R-5-P it is not likely that the pyruvate from 6-PG+TPN was produced through the pentose cycle, and the origin of this additional pyruvate therefore remains unexplained at present.

Pentose Phosphate Utilization

Ribose-5-phosphate (R-5-P) was degraded readily by sonic preparations of *A. melanogenum*. The disappearance of this pentose was accompanied by the production of a compound resembling sedoheptulose (11). It may be noted in Fig. 3 that the absorption peak at 670 mμ characteristic of the orcinol reaction with pentose disappeared and a broad band of low intensity between

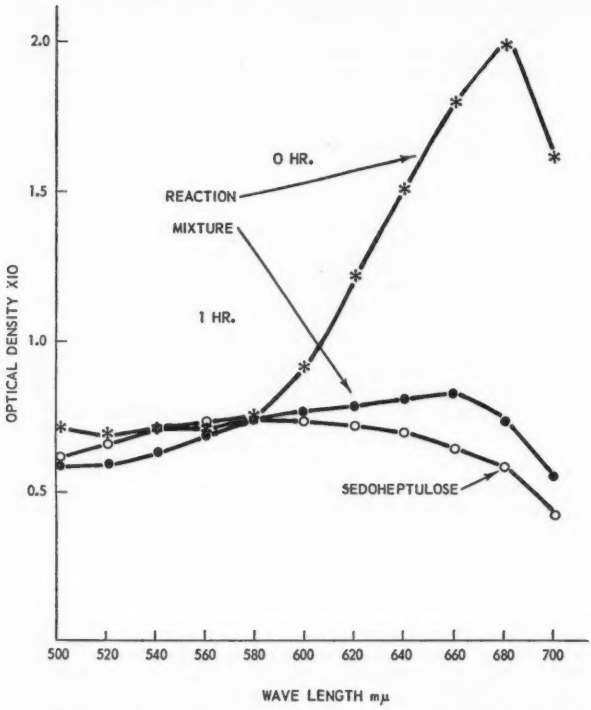

FIG. 3. Utilization of ribose-5-phosphate by a sonic extract of *A. melanogenum* as demonstrated by the orcinol reaction spectrum. The reaction mixture contained: 3 ml. of crude enzyme and 3 ml. of R-5-P (50 μM./ml.); incubation temperature 30° C.; 1 ml. aliquots removed, acidified with 0.2 ml. 40% trichloracetic acid, and centrifuged; 0.01 ml. of supernatant assayed for pentose with the orcinol–$FeCl_3$ reagent; sedoheptulosan was used to obtain the spectrum of sedoheptulose.

580 and 670 mμ appeared; this is similar to that produced by the heptulose (11). In Table IV are given results of analyses of an orcinol reaction mixture with R-5-P as substrate. The amounts of pentose and heptulose were calculated by solution of simultaneous equations (12). It is clear that pentose disappeared whereas a sedoheptulose-like compound appeared. However, the

TABLE IV

RIBOSE-5-PHOSPHATE UTILIZATION BY AN EXTRACT OF *Acetobacter melanogenum**

Minutes	Pentose (μg.)	Heptulose (μg.)
0	23.0	0.8
10	18.8	3.0
30	12.1	4.4
60	8.8	3.6

*The reaction mixture is given in Fig. 3.

latter did not accumulate to any extent. When the products of this experiment were tested spectrophotometrically in the presence of TPN rapid reduction took place. That this was likely due to the presence of G-6-P was shown further by heating the sample and using TPN plus commercial Zwischenferment; again rapid TPN reduction could be demonstrated. On dephosphorylation of the 60-minute mixture with potato phosphatase and deionizing on a mixed Dowex 2 - Dowex 50 column a solution was obtained which was found by means of paper chromatography (with water-saturated phenol as solvent), to contain fructose, glucose, ribose, and sedoheptulose. These results suggest the operation of the pentose cycle via the transketolase and transaldolase series of reactions (9).

Kinases and Other Enzymes

Numerous attempts were made to demonstrate the presence of hexokinase, gluconokinase, 2-ketogluconokinase, and phosphohexokinase activity in the

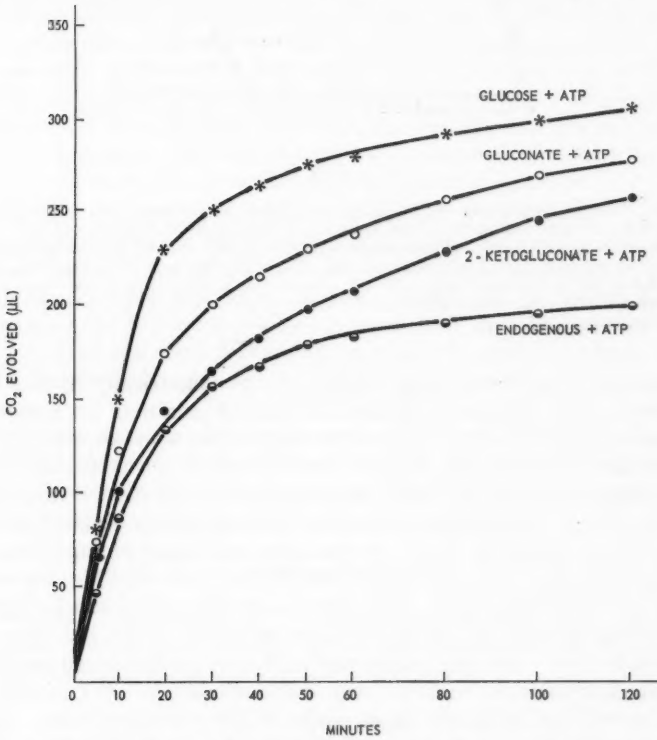

FIG. 4. Kinase activity of *A. melanogenum*. The Warburg vessels contained: 2.0 ml. sonic extract (pH 6.0); 0.5 ml. $NaHCO_3$ (*M*/10); 0.1 ml. $MgCl_2$ (*M*/5) in main compartment; 0.2 ml. ATP (*M*/25), 0.2 ml. $NaHCO_3$ (*M*/25) in one side arm; 0.2 ml. substrate (*M*/5), 0.2 ml. $NaHCO_3$ (*M*/25) in the second side arm; 30° C. Gas phase: N_2 95%–CO_2 5%.

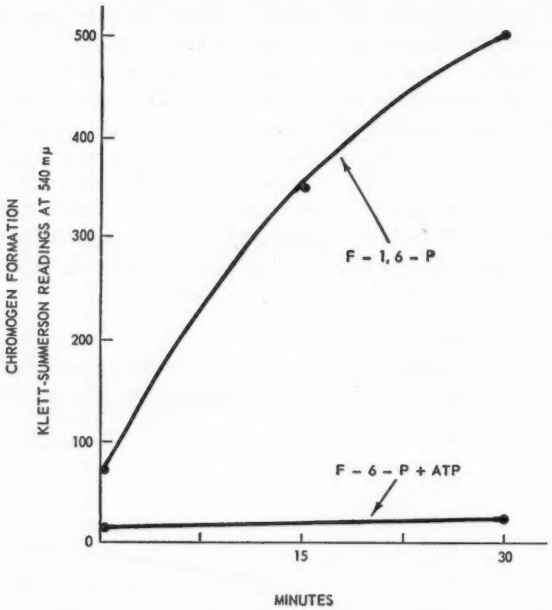

FIG. 5. Phosphohexokinase and aldolase activity of an extract of *A. melanogenum*. The reaction mixture contained: 0.5 ml. sonate; 1.0 ml. tris buffer (*M*/10, pH 8.6); 0.25 ml. fructose-1,6-diphosphate or fructose-6-phosphate (*M*/20); 0.25 ml. hydrazine sulphate (0.56 *M*) and water to a total volume of 5.0 ml. ATP (20 μM.) and $MgCl_2$ (0.2 ml., *M*/5) were added to the mixture containing F-6-P. One milliliter aliquots were removed at the time intervals indicated, deproteinized with trichloracetic acid, and analyzed for "chromogen" formation (18).

sonic extracts. These tests were carried out manometrically by the bicarbonate method of Colowick and Kalckar (1). In addition the presence of hexokinase and gluconokinase was determined spectrophotometrically with adenosinetriphosphate (ATP) and TPN. Kinases for gluconate and 2-ketogluconate were tested also with ATP using pyruvate production as the criterion of activity (17). Phosphohexokinase was determined by linking with the active aldolase present in these preparations and determining chromogen production (from fructose-6-phosphate (F-6-P) Mg^{++} and ATP) by the method of Sibley and Lehninger (18). The presence of glyceraldehyde-3-phosphate (G-3-P) dehydrogenase was determined by measuring DPN reduction with G-3-P as substrate. As may be observed in Fig. 4, hexokinase, gluconokinase, and 2-ketogluconokinase activity was demonstrable by the bicarbonate method, hexokinase being the most active of the three enzymes. Strong ATP-ase activity was also present. TPN reduction in the presence of ATP and Mg^{++} was obtained only with glucose; however, an active gluconokinase was demonstrated using pyruvate production as the criterion, the system in this case containing gluconate, ATP, and Mg^{++}. Phosphohexokinase activity

was not obtained with the method employed although under the same conditions a very active aldolase was demonstrable (Fig. 5). DPN reduction occurred with pure G-3-P, indicating the presence of G-3-P dehydrogenase, although the rate of DPN reduction was not rapid. This may be due to the fact that no attempt was made to prevent the reoxidation of the DPNH produced in this reaction.

Discussion

It is clear that *A. melanogenum* possesses separate systems for the oxidation of glucose and hexose phosphates. In this respect it closely resembles *A. suboxydans* (10), *P. fluorescens* (20, 21), and a variety of other organisms. *A. melanogenum*, however, appears to be capable of carrying the non-phosphorylative oxidation of glucose at least several steps further by oxidizing 2-ketogluconate to a postulated 2,5-diketogluconate (3, 13) and the latter intermediate through a series of reactions to α-ketoglutarate (4). However, it is not possible to decide from the available data which system is operative in this organism; both may be utilized since hexokinase appears to be present. The situation is similar to that reported for *A. suboxydans* which oxidizes glucose directly and possesses an active hexokinase (6, 7). However, it would seem reasonable to conclude from the data available that the oxidative rather than the glycolytic pathway is used by *A. melanogenum* since phosphohexokinase activity could not be demonstrated in this organism.

Oxidation through the phosphorylative route may be achieved in several ways after 6-PG is produced from G-6-P. The former compound may be utilized further through the pentose cycle or may undergo a C_3—C_3 split yielding pyruvate and triose phosphate. Under the conditions of the above experiments the latter course appears more likely as considerably more pyruvate was produced from 6-PG than from R-5-P, whereas approximately the same amount of pyruvate would be expected if the 6-PG were first converted to pentose. The origin of pyruvate from R-5-P may be by way of the transketolase–transaldolase series of reactions leading to F-6-P, G-6-P, and then to 6-PG followed by a C_3—C_3 split. Or it may be produced from triose phosphate originating from the pentose phosphate itself through the transketolase reaction. Both pathways may be operative.

Acknowledgment

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THE CHEMICAL COMPOSITION OF THE ACTINOMYCETALES: ISOLATION OF A POLYSACCHARIDE CONTAINING D-ARABINOSE AND D-GALACTOSE FROM NOCARDIA ASTEROIDES[1]

C. T. Bishop and F. Blank

Abstract

A polysaccharide isolated from cultures of *Nocardia asteroides* contained arabinose and galactose in a molar ratio of 1.7:1. The two monosaccharides were unequivocally identified as D-arabinose and D-galactose by isolating them in crystalline form. Partial hydrolysis showed that some of the D-arabinose units in the polysaccharide were in the furanoside ring form while the D-galactose units possessed the pyranoside structure. Methylation studies showed that the polysaccharide was a branched structure of D-arabinose and D-galactose units with some of the arabinose forming non-reducing, terminal residues. The findings reported here are regarded as further evidence of the close taxonomic relationship between *Nocardia asteroides* and *Mycobacterium tuberculosis*, members of the same order.

Introduction

In an investigation by Avery and Blank (2) neither chitin nor cellulose could be isolated from six species of four different genera of the Actinomycetales (*a*) *Actinomyces bovis*, (*b*) *Actinomyces israeli*, (*c*) *Nocardia asteroides*, (*d*) *Streptomyces albus*, (*e*) *Streptomyces griseus*, and (*f*) *Micromonospora chalcea*. These results, together with a number of morphological and physiological features, were interpreted as indicating that the Actinomycetales belong to the bacteria rather than the fungi. After completion of the foregoing investigation it was felt that examination of the species mentioned for polysaccharides other than chitin and cellulose might furnish some biochemical evidence about the taxonomic relationship between the Actinomycetales and other microorganisms. The present report describes the isolation and investigation of a polysaccharide from *Nocardia asteroides*.

Experimental

Paper chromatography was carried out by the descending method using the following solvent systems (v/v ratio):

(A) Pyridine:ethyl acetate:water—1:2:2 (12).
(B) Benzene:amyl alcohol:ethanol:water—1.7:1.0:1.0:0.25.
(C) Butanol:pyridine:water—6:2:3 (10).
(D) Butanol:ethanol:water—40:11:19 (10).

Unsubstituted sugars were detected on chromatograms by silver nitrate: sodium hydroxide sprays (21). Methyl ethers of monosaccharides were detected by *p*-anisidine hydrochloride spray reagent (10). Evaporations were carried out under reduced pressure at 35° C. or less. Melting points are corrected and specific rotations are equilibrium values unless otherwise stated.

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Isolation of Polysaccharide

Nocardia asteroides was cultured on a modified Sabouraud glucose agar (2) at 37° C. Cultures were autoclaved at 121° C. for 20 minutes and the growth was collected by filtration of the hot liquid medium. The cells were washed several times on the filter with hot water and then were boiled with water three times, being separated each time by centrifuging. The washed cells were then freeze-dried and powdered in a ball mill. The dry product (30 g.) was extracted continuously with petroleum ether (b.p. 30–60° C.) for 72 hours, the extraction removing 8 g. of lipid material which was discarded. The residue from this extraction contained 8.66% nitrogen. A number of residues from the preceding treatments were combined (40 g.) and digested with trypsin (0.16 g.) in a carbonate buffer (345 ml.) at pH 8.2 containing 0.7 ml. of chloroform. After 2 weeks at 37° C. the insoluble material was centrifuged, freeze-dried, and incubated again with trypsin. This procedure reduced the nitrogen content to 4% and this product (32 g.) was refluxed with 3% aqueous sodium hydroxide (640 ml.) for 14 hours. The solution was neutralized with acetic acid, clarified by centrifuging, and the supernatant liquor was dialyzed through cellophane bags against running tap water for 48 hours. Addition of two volumes of ethanol to the solution of non-dialyzable material caused the precipitation of polysaccharides which were reprecipitated once, washed with ethanol, then ether, and finally dried *in vacuo* at 20° C. The mother liquors from the above precipitations deposited more of the same material when cooled to 5° C. The polysaccharide preparation obtained in this way was a gray-brown powder. Analysis: Calc. range for a polysaccharide: C, 44.4–45.5%; H, 6.07–6.18%. Found: C, 44.54%; H, 6.43%; N $< 0.5\%$.

Purification of Polysaccharide

Aqueous solutions of the above polysaccharide preparation were very highly colored and polarimetric readings could not be obtained on 1 to 2% solutions. This color could not be removed by extraction with chloroform, ether, ethyl acetate, or butanol, and was not absorbed on charcoal or the ion exchange resins Amberlite IR-120 or Amberlite IR-4B. The polysaccharide preparation (13 g.) was therefore oxidized by chlorite at pH 5 and 60° C. for 4 hours (23). This procedure changed the color of the solution from dark brown to yellow. The oxidation mixture was dialyzed through cellophane against tap water for $2\frac{1}{2}$ hours and against distilled water for 48 hours. The solution of non-dialyzable material was concentrated to one-fourth of its volume and addition of four volumes of ethanol precipitated polysaccharide material which was washed successively with absolute ethanol, ether, and petroleum ether before being dried *in vacuo* at 20° C. This product was now insoluble in water but soluble in alkali. It was a white powder having $[\alpha]_D^{27} +26 \pm 2°$ (*c*, 1.8% in 4% sodium hydroxide) and a number average degree of polymerization of 26 ± 2, based on estimation of reducing end group by hypoiodite oxidation using a phosphate buffer at pH 11.9 (3).

Estimation of Component Sugars

A sample (50 mg.) of the polysaccharide was heated at 97° C. with *N* hydrochloric acid (2 ml.) for 11 hours. Paper chromatography of the hydrolyzate (solvent A) showed the presence of components having the same mobilities as known samples of galactose, arabinose, and glucose, run on the same paper sheet. Quantitative estimation of these sugars (9) gave a molar ratio of arabinose:galactose of 1.7:1. The glucose was present in only trace amounts, too small to be determined.

Partial Hydrolysis

The polysaccharide (15 mg.) was heated at 97° C. for 4½ hours with 0.025 *N* oxalic acid and the hydrolyzate was examined by paper chromatography (solvent A). The hydrolyzate contained primarily arabinose with a faint trace of galactose being detected.

Identification of Component Sugars

A sample (0.50 g.) of the polysaccharide was heated with *N* hydrochloric acid (20 ml.) at 97° C. for 11 hours. An insoluble residue (70 mg., 14%) was removed by centrifuging and the hydrolyzate was neutralized by Amberlite IR-4B. Evaporation of the neutral solution left the mixture of monosaccharides as a yellow sirup (324 mg.). This mixture was resolved by partition chromatography on a column of Celite (3.75×34 cm.) using *n*-butanol, saturated with water, as the developing phase (15). The eluate was collected in 3 ml. fractions by an automatic fraction collector which changed the receiver every 3 minutes. A preliminary 500 ml. of eluate was run off before the column was mounted on the fraction collector. Every fifth fraction was examined by paper chromatography (solvent A) and fractions containing the same single component were combined. Fractions 10–112 contained arabinose, Fractions 113–149 contained arabinose and galactose, Fractions 150–263 contained galactose and since this was the last sugar on the column its elution was completed by running a further 200 ml. of eluate directly into a graduate.

Fractions 10–112, containing only arabinose, were combined and evaporated to dryness. The residue was dissolved in warm methanol, decolorized by charcoal, and filtered. When the filtrate was allowed to evaporate slowly in a desiccator the arabinose crystallized, m.p. 152–155° C. raised to 154–155° C. by one recrystallization from methanol. The specific rotation of this compound, $[\alpha]_D^{25}$ $-105° \pm 2°$ (*c*, 0.9% in water), showed that it was D-arabinose and not the more common L-isomer. The crystalline D-arabinose was recrystallized from glacial acetic acid to a constant m.p. 156–156.5° C. A mixed melting point of this product with L-arabinose was depressed to 150–151° C. while a mixed melting point with an authentic sample of D-arabinose was 156–156.5° C.

Fractions 150–263, together with the extra 200 ml., contained only galactose and were evaporated to dryness. The residue, in water, was decolorized by charcoal and slow evaporation of the filtrate in a desiccator left a crystalline

residue. Recrystallization from glacial acetic acid yielded α-D-galactose, m.p. 167–169° C., $[\alpha]_D^{26}$ +100°±2° (5 minutes) → +81°±2° in 4 hours (*c*, 0.2% in water).

Periodate Oxidation

Two samples (120 mg. and 127 mg.) of the polysaccharide were each shaken with a solution of sodium metaperiodate (1.2 g.) in water (145 ml.). The mixtures and two blanks were shaken continuously at room temperature in the absence of light. At the times shown below samples were removed from each flask for estimation of formic acid and periodate. Formic acid was determined, after destruction of excess periodate by 2,3-butanediol, by titration of the iodine liberated from potassium iodide. Periodate was determined by the excess arsenite method (6, 7). The results, given in moles per mole anhydroarabinose unit, were as follows:

Time (hr.)	22	80	144	236
Formic acid	0.20	0.27	0.29	0.32
Periodate	0.60	0.72	0.72	0.79

Methylation

The polysaccharide preparation (5 g.) was stirred in water (30 ml.) and methylated by the dropwise addition of dimethyl sulphate (50 ml.) and 40% sodium hydroxide (100 ml.). These reagents were added at such a rate that the mixture was neutral for the first hour and alkaline thereafter. After addition of reagents was complete the mixture was stirred overnight and then another methylation was carried out using the same quantities of reagents. The mixture was neutralized with glacial acetic acid and dialyzed against distilled water for 60 hours. The solution of non-dialyzable material was freeze-dried to yield the partially methylated polysaccharide (3.9 g.) which still showed a predominant hydroxyl band at 3500–3600 $cm.^{-1}$ in its infrared spectrum. The partially methylated product was then stirred into tetrahydrofuran (100 ml.) and sodium hydroxide pellets (30 g.) were added. Methylation was carried out by dropwise addition of dimethyl sulphate (40 ml.) to the stirred mixture over a period of 4 hours. Stirring was continued for a further 20 hours, after which sufficient water was added to dissolve the sodium salts. Excess dimethyl sulphate and tetrahydrofuran were removed by evaporation. The alkaline aqueous mixture was neutralized with glacial acetic acid and extracted five times with fresh one-half volumes of chloroform. The chloroform was dried over anhydrous sodium sulphate and evaporated to give a brown, glassy solid (2.4 g.) which still showed a hydroxyl band in its infrared spectrum. The product was therefore methylated again in tetrahydrofuran as just described to yield the fully methylated polysaccharide (2.2 g.) having no hydroxyl band in its infrared spectrum and containing 38.2% methoxyl groups.

The methylated product was fractionated by extraction with petroleum ether containing increasing amounts of chloroform. In this way fractions were

obtained using petroleum ether:chloroform ratios of 90:10, 80:20, 70:30, 60:40, and 50:50. These fractions totaled 2.04 g. and therefore constituted the major portion of the methylated product. Each fraction showed a specific rotation of $[\alpha]_D^{28}$ $+31° \pm 3°$ (*c*, 1% in chloroform) and on hydrolysis and chromatography (solvents B and D) each showed the same cleavage products. For these reasons the fractions were combined to give 2.0 g. of fully methylated, apparently homogeneous, polysaccharide.

Hydrolysis of Methylated Polysaccharide and Separation of Hydrolysis Products

The methylated polysaccharide (2.0 g.) was refluxed with 7% methanolic hydrogen chloride (100 ml.) for 10 hours. Methanol was evaporated at room temperature by a stream of air and the residue was taken up in *N* hydrochloric acid. This solution was heated on a boiling water bath for 7 hours. Acid was removed by Amberlite IR-4B resin and the neutral hydrolyzate was evaporated to yield a sirup which was dried at 5 mm. over anhydrous calcium chloride, yield 1.14 g.

Paper chromatography of this sirup (solvents B, C, and D) showed the presence of 2,3,5-tri-*O*-methyl-D-arabinose; 2,3,6-tri-*O*-methyl-D-galactose; a di-*O*-methyl-D-arabinose; 2,3,4-tri-*O*-methyl-D-galactose; 2,6-di-*O*-methyl-D-galactose; and a mono-*O*-methyl-D-arabinose. The 2,3,6-tri-*O*-methyl-D-galactose was the major component. The methylated aldopentoses were distinguished from the methylated aldohexoses by their color with the *p*-anisidine spray reagent (10). The configurations of these compounds were assumed to be the same as those found for the unsubstituted monosaccharides. The mixture of monosaccharide methyl ethers was partially resolved by partition chromatography on a column of Celite (15). This procedure gave four fractions, each of which still contained at least two compounds. Pure specimens of each of the monosaccharide methyl ethers were obtained by paper sheet chromatography of each fraction from the Celite column. Solvents B, C, and D were used depending on which one gave the best separation of components in a particular fraction. The methylated sugars so isolated were contaminated with an oily product from which they were separated by extraction with ethyl acetate.

Identification of Components

The pure specimens of the monosaccharide methyl ethers were characterized by isolating them in a crystalline form or by converting them to crystalline derivatives. The 2,3,5-tri-*O*-methyl-D-arabinose was converted to 2,3,5-tri-*O*-methyl-D-arabonamide (11), m.p. 133–134° C., $[\alpha]_D^{28}$ $-22° \pm 2°$ (*c*, 1% in ethanol). 2,3,6-Tri-*O*-methyl-D-galactose was identified in the same way as 2,3,6-tri-*O*-methyl-D-galactonamide (1), m.p. 129–131° C. The 2,3,4-tri-*O*-methyl-D-galactose was refluxed with aniline in ethanol solution giving the characteristic 2,3,4-tri-*O*-methyl-*N*-phenyl-D-galactosylamine (16, 19), m.p. 166–168° C., $[\alpha]_D^{28}$ $-68° \pm 3° \rightarrow +42° \pm 3°$ in 5 hours (*c*, 1% in methanol). The 2,6-di-*O*-methyl-D-galactose crystallized when nucleated with an authentic specimen of this compound. Recrystallization from acetone:petroleum ether

gave 2,6-di-*O*-methyl-β-D-galactose monohydrate (13), m.p. 99–100° C., $[\alpha]_D^{26}$ $+61° \pm 2° \rightarrow +84° \pm 2$ in 3 hours (*c*, 1% in water). The di-*O*-methyl and mono-*O*-methyl-D-arabinose failed to crystallize nor could crystalline anilides or phenylhydrazones be obtained.

Discussion

A crude highly colored polysaccharide preparation was isolated from defatted, deproteinized cells by alkali extraction. The color was destroyed by chlorite oxidation (23) leaving the polysaccharide as a white powder. This material had a specific rotation of $[\alpha]_D^{27}$ $+26°$, was insoluble in water but soluble in alkali, and had a number average degree of polymerization of 26 ± 2. It should be emphasized that this must be regarded as the minimum size of the polysaccharide because of probable degradation during the vigorous treatments with alkali (22) and chlorite (20). The polysaccharide as it existed *in situ* was probably much larger.

Hydrolysis of the polysaccharide yielded galactose and arabinose in a molar ratio of 1:1.7. The two monosaccharides were unequivocally identified as D-galactose and D-arabinose by isolation as crystalline products, the melting points and specific rotations of which agreed with those of authentic samples. The identification of arabinose in the form of its D-isomer was extremely interesting because this sugar has rarely been found in natural products. Naturally occurring D-arabinose was first reported by Léger (14), who found that it formed the glycone moiety of an aromatic glycoside in aloes. It was later found as a component of the cleavage products of the wax of *Mycobacterium tuberculosis* (8, 17). Partial hydrolysis of the polysaccharide from *N. asteroides* by dilute oxalic acid released primarily arabinose with traces of galactose. These results showed that some of the D-arabinose units were in the furanoside form while most, if not all, of the D-galactose units possessed the pyranoside structure. The polysaccharide was converted to its methyl ether, which was then subjected to solvent fractionation. The fractions so obtained were similar in specific rotations and hydrolysis products so they were recombined and hydrolyzed to yield the following monosaccharide methyl ethers:

2,3,5-tri-*O*-methyl-D-arabinose, 2,6-di-*O*-methyl-D-galactose,
2,3,6-tri-*O*-methyl-D-galactose, a di-*O*-methyl-D-arabinose
2,3,4-tri-*O*-methyl-D-galactose, a mono-*O*-methyl-D-arabinose } unidentified.

These compounds were identified by their position on paper chromatograms and by their color reactions with the *p*-anisidine hydrochloride spray (10). Identities of the first four compounds listed were confirmed by isolating them in a crystalline form or by preparing crystalline derivatives. To effect these identifications the mixture was partially resolved by chromatography on a Celite column (15) and pure specimens were finally obtained by paper sheet chromatography. These separations were difficult to achieve because of an oily impurity that appeared to cause the zones of different sugars to overlap on the chromatograms. Final purification of the separated sugars was achieved by extracting them with ethyl acetate in which the oily impurity was insoluble. It is probable, because of the low results on periodate oxidation

(see below), that the original polysaccharide was contaminated by some other product that gave rise to this oily material on methylation. Because of these difficulties in separating the monosaccharide methyl ethers, no reliable quantitative estimate of them could be made. However, from visual inspection of chromatograms of the mixture, it was apparent that 2,3,6-tri-*O*-methyl-D-galactose was the largest component. Results of the methylation study therefore showed that a large number of the D-galactose units were linked through carbons 1 and 4 with others being joined through carbons 1 and 6, and through carbons 1, 3, and 4. The only terminal units found were D-arabinose, in the furanose form, indicating that the two sugars, D-arabinose and D-galactose, were joined together in the same polysaccharide. This substantiated the evidence for homogeneity of the polysaccharide arising from the lack of any distinct separations by fractionation of the methylated polysaccharide. That some of the D-arabinose units were non-terminal was shown by the presence of a dimethyl and monomethyl ether of that sugar.

The polysaccharide when oxidized by periodate consumed 0.79 moles of oxidant per mole of anhydroarabinose with a concurrent production of 0.32 moles of formic acid. With the limited amount of quantitative information obtained from the methylation study the periodate results cannot be interpreted unequivocally. The formic acid could be produced only from monosaccharide units containing three vicinal hydroxyl groups and hence, in this polysaccharide, must have come from the $1 \rightarrow 6$ linked D-galactose units. Theoretically, 1 mole of periodate is consumed by every monosaccharide unit possessing two vicinal hydroxyl groups. On this basis, all polysaccharides except $1 \rightarrow 3$ linked hexosans and $1 \rightarrow 2$ or $1 \rightarrow 3$ linked furanoside pentosans should consume at least 1 mole of periodate per mole of monosaccharide unit. The low value observed for the polysaccharide investigated here may have been caused by the impurity mentioned in connection with the methylation data.

Although the polysaccharide containing D-arabinose and D-galactose was isolated from *Nocardia asteroides* here in an impure state, it was the only polysaccharide that could be found under these conditions and most probably forms the skeleton of the cell wall. The presence of a cell wall polysaccharide in *Nocardia* species has been reported recently by Romano and Sohler (18). These authors tentatively identified arabinose and galactose as components of this material but the configurations of these sugars were not determined. The absence of chitin and cellulose in the Actinomycetales, together with a number of morphological and physiological features of these organisms, had led to the conclusion (2) that the Actinomycetales had nothing in common with the Eumycetes and were more closely related to certain groups of bacteria, such as the *Corynebacteria*, than to the fungi. Cummins and Harris (4, 5) have reported the chromatographic detection of galactose and arabinose in hydrolyzates of the cell walls of *Mycobacterium*, *Nocardia*, and *Corynebacterium* with the latter also containing mannose. To our knowledge, D-arabinose has only been found once before in microorganisms, i.e. as a component of the wax of *Mycobacterium tuberculosis* (8, 17). Based on comparative biochemistry,

the finding of a polysaccharide containing D-arabinose and D-galactose in *Nocardia asteroides* supports the classification of this organism and *Mycobacterium tuberculosis* as members of the same order. It also demonstrates the close taxonomic relationship of these two organisms.

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CARBOHYDRATE METABOLISM OF PULLULARIA PULLULANS[1]

D. S. CLARK[2] AND R. H. WALLACE

Abstract

Pullularia pullulans, ATCC No. 11942, has constitutive enzymes for the utilization of galactose, glucose, maltose, mannose, raffinose, sucrose, and trehalose, but not for lactose. The organism oxidized mannose more rapidly, and maltose and raffinose more slowly, than any of the other sugars. Moreover, it utilized galactose and glucose at approximately the same rate, but more rapidly than trehalose; the oxidation of sucrose, however, was very slow at first and then very rapid. When resting cells were incubated with glucose-1-C^{14}, the carbon dioxide immediately produced was highly radioactive; when glucose-6-C^{14} was used, however, a considerable time elapsed before the production of noticeable amounts of radioactive carbon dioxide. The ratio of the activity of the carbon dioxide produced from glucose-1-C^{14} to that produced from glucose-6-C^{14} was greater in young cells than in old. The results indicated that resting cells catabolize glucose in such a manner that most of the carbon dioxide comes from carbon 1 of glucose. Extracts from cells were found to have an active phosphoglucomutase, glucose-6-phosphate dehydrogenase, 3-phosphoglyceraldehyde dehydrogenase, and fructose diphosphate aldolase. Both dehydrogenases were TPN-linked. The extracts also oxidized ribose-5-phosphate but not ribose. The organism has enzymes, therefore, to catabolize glucose by some of the reactions of the Embden–Meyerhof system as well as by those of the pentose phosphate cycle.

Introduction

Pullularia pullulans is a yeast-like fungus that is widely distributed in nature. Numerous references (4) concerning the ecology of the organism show that its occurrence is not limited to a specific location nor to specific material. They also indicate that the organism is important industrially in that it causes paint deterioration, discoloration of lumber products, and is the primary, secondary, or co-operating organism causing injury to many plants and plant products.

A few investigators have studied certain aspects of the physiology of *P. pullulans*. Fraser (5) included the organism in a study of the nutritional requirements of a number of sooty molds. He reported that *P. pullulans* grows rapidly in media containing inulin, dextrin, and starch; that it grows slowly in media containing pentose, mannose, and lactose; and that it does not utilize raffinose as a carbon source. Bauer (1) found that conidia of the organism develop best in media containing starch, mannitol, glycerol, fructose, alcohol, and dextrose. Negroni and Fischer (10), from a study of physiological characteristics of 30 strains of *P. pullulans*, reported that they all utilize dextrose, maltose, lactose, raffinose, and galactose as sources of carbon.

Apparently very little is known about the intermediate metabolism of *P. pullulans*. It was considered timely, therefore, to conduct studies that

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might explain some of the metabolic activities of the organism. An investigation was made at the outset to determine if the organism has adaptive or constitutive enzymes for the utilization of various sugars; the purpose of subsequent work was to establish the rates of oxygen uptake by the organism in the various intermediary compounds of glucose metabolism, and to estimate the way in which the organism metabolizes carbohydrates.

Experimental

I. Utilization of Carbohydrates

Methods

Conidia of *P. pullulans*, ATCC No. 11942, were grown at room temperature, with moderate aeration, and in media having the following ingredients: ammonium nitrate, 0.1%; magnesium sulphate, 0.05%; potassium monohydrogen phosphate, 0.1%; yeast extract (Difco), 0.1%; and one of several sugars, 2%. Each of the following sugars was used as a carbon source: galactose, glucose, lactose, maltose, mannose, raffinose, sucrose, and trehalose. Each medium was sterilized by means of Seitz filtration.

The cells were centrifuged from their growth media and then washed three times with distilled water. The quantity of distilled water for each washing was 10 times the volume of the cells. The washed cells were resuspended in 0.1 *M* potassium dihydrogen phosphate buffered at pH 6.8 and were aerated for 2 hours with a rapid stream of sterile air to decrease the level of endogenous respiration. The aerated cells were centrifuged from the liquid phase of the suspension and resuspended in a fresh supply of phosphate buffer; enough buffer was added so that 1 ml. of suspension in 10 ml. of distilled water would record 45% light transmission with a Fisher electrophotometer. The number of viable cells per ml. of suspension was determined by means of plating.

The following method was used to detect a possible lag in the uptake of oxygen during the utilization of the various substrates, and to indicate therefore, the existence of adaptive enzymes. Cells that had been grown for 15 days in a medium containing a specific sugar, e.g., glucose, were transferred to three media; one medium contained the specific sugar being tested and each of the others contained a different sugar. These were moderately aerated for 6 days and then 5 ml. of each were transferred to a corresponding fresh medium to be aerated again for 6 days. A portion of each of the resulting suspensions was centrifuged, washed, aerated, and adjusted to a standard density. Three 1 ml. aliquots of each new suspension were then placed in reaction vessels containing the original sugar in which the cells had been grown, e.g., glucose, and the rates of oxygen uptake were recorded.

Conventional manometric techniques were used to measure the rates of respiration of the cells (13). The temperature of the water bath of the Warburg apparatus was 30° C., and the agitation speed was maintained as closely as possible at one hundred and twenty 2-in. strokes per minute. In all instances the gas phase was air and the pH of the reaction mixture was 6.8. All respiration tests were done in triplicate.

Results

In general very little variation occurred between triplicate tests. Cells of *P. pullulans* were capable of utilizing all of the sugars tested except lactose, but there were differences in the rates of utilization. Table I shows that the cells consumed oxygen more rapidly in the presence of mannose than they did with any of the other sugars and that they oxidized maltose and raffinose the slowest. It appears also that they utilized galactose and glucose at approximately the same rate, but more rapidly than trehalose; and that their oxygen uptake with sucrose was very slow during the first 40 minutes but very rapid after that time. A number of attempts were made to "train" the cells to use lactose as a carbon source but none were successful.

TABLE I

OXIDATION OF SUGARS BY *P. pullulans*

	μl. oxygen consumed per 100 million cells						
Minutes	Galactose	Glucose	Maltose	Mannose	Raffinose	Sucrose	Trehalose
20	49	42	22	48	21	31	32
40	103	99	49	112	47	40	78
60	151	155	86	179	81	141	118
80	209	212	124	276	121	221	162
100	—	264	156	319	174	—	202
120	—	—	190	—	267	—	246

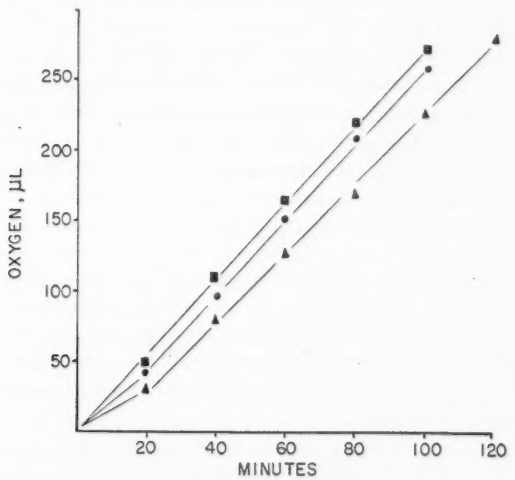

FIG. 1. Utilization of glucose per 100 million cells of *P. pullulans*. ■—■ mannose-grown cells; ●—● glucose-grown cells; ▲—▲ maltose-grown cells. Main compartments of test reaction-vessels contained 1 ml. of cell suspension, 0.5 ml. of substrate (0.08 *M*), and 1 ml. of phosphate buffer (0.1 *M*). Center wells of all vessels contained 0.2 ml. of 20% potassium hydroxide. Dry weight of cells per ml. of glucose-grown cells, 5.4 mg.; of mannose-grown cells, 5.4 mg.; and of maltose-grown cells, 5.0 mg.

Fig. 1 shows the rates of oxygen uptake of cells with glucose as the substrate; the curves represent cells that had been previously cultured in mannose, glucose, and maltose, respectively, but which had been originally cultured in glucose. The rates of oxidation were typical for all the other sugars, except lactose, in that there was not, in any instance, a distinct lag in the initial rate of uptake of oxygen.

II. Metabolism of Glucose by Intact Cells

(In collaboration with Professor W. F. Oliver, Physics Department, Macdonald College)

In recent years there has been an increasing amount of evidence to show that the pentose phosphate oxidative scheme, with a direct oxidation of glucose-6-phosphate, is of fundamental significance in the metabolism of certain microorganisms and tissues. It has also been established that some microorganisms catabolize glucose by means of a combination of direct oxidation and the reactions that take place according to the Embden–Meyerhof system (2, 8). Many of the schemes for the oxidative metabolism of glucose have the common feature that carbon dioxide is produced largely from the first carbon atom of glucose. They differ, therefore, from the Embden–Meyerhof scheme in which the carboxyl groups of pyruvic acid are derived from the third and fourth carbon atoms of glucose. This preferential release of carbon dioxide makes it possible for investigators to obtain evidence, by means of radioactive compounds, of the presence of an oxidative scheme of glucose catabolism without the necessity of identifying intermediate compounds and end-products. It was decided, therefore, to use glucose-1-C^{14} and glucose-6-C^{14} in an attempt to show if resting cells of *Pullularia pullulans* oxidize glucose through the intermediates of the pentose phosphate scheme, and if the age of the cells has any effect on the amount of glucose that may be metabolized in this manner. Experiments of this nature have not been reported for *P. pullulans* or with any other yeast-like fungus.

Methods

P. pullulans was grown with strong aeration for 6, 18, 36, and 108 hours, respectively, at room temperature, in a medium consisting of ammonium nitrate, 0.1%; casein hydrolyzate, 0.1%; glucose, 1%; magnesium sulphate, 0.05%; potassium monohydrogen phosphate, 0.1%; and yeast extract, 0.2%, adjusted to pH 6.8. The cells were harvested, washed, and aerated in the manner previously described.

Enzymatic activity of the washed cells was determined by conventional manometric methods (13) at 30° C. and at an agitation speed of one hundred and twenty 2-in. strokes per minute. The main compartments of the test reaction-vessels contained 3 ml. of cell suspension in 0.1 *M* phosphate buffer (pH 6.8). The center wells of all vessels contained 0.2 ml. of 20% carbonate-free potassium hydroxide. The substrates, glucose-1-C^{14} and glucose-6-C^{14},

having a final molarity of 0.0143 were first placed in 0.5 ml. quantities in the side arms and later, after a 10 minute adjustment time, tipped into the main compartments. In the control vessels the glucose was replaced with water. At intervals of $\frac{1}{2}$, 1, 2, 3, and 5 hours after the addition of the substrate two flasks containing glucose-1-C^{14} and glucose-6-C^{14}, respectively, were removed. From the center wells, 0.1 ml. portions were pipetted into separate 10 ml. volumetric flasks. The contents of each flask were made up to volume with water and thoroughly mixed. Three 2 ml. aliquots were then pipetted from each volumetric flask onto separate stainless steel disks (1.5 cm. in diameter) and allowed to dry on a level surface at 60° C. The activities of the samples were measured with a mica window Geiger counter, corrections being made for background emissions and circuit variations. Corrections for self-absorption were unnecessary since the thickness of each sample was constant. In all instances the maximum counting error was less than 3%.

Glucose-1-C^{14} was obtained from Atomic Energy of Canada Limited and glucose-6-C^{14}, from Tracerlab Incorporated. Stock solutions were prepared by adding non-radioactive glucose and 0.1 *M* phosphate buffer to each sugar; 0.5 ml. of each solution contained 9 mg. of glucose and produced approximately 1,000,000 disintegrations per minute. A measurement of the relative radioactivity of equal portions of the sugar stock solutions showed that the C-1^{14}/C-6^{14} averaged 1.0526. Assuming that all of the labelled material was at carbon 1 and carbon 6, respectively, all the values obtained for glucose-6-C^{14} were multiplied by that figure so that they would be comparable with those for glucose-1-C^{14}.

Results

Measurements were made for oxygen consumption in all tests to find if glucose-1-C^{14} and glucose-6-C^{14} were oxidized at the same rate. Table II shows decisively that cells of *P. pullulans* did not prefer one type of glucose to the other. Table II also shows that resting, 6-hour-old cells of *P. pullulans* produced, in the first 30 minutes of incubation, 14 times as much carbon dioxide from carbon 1 of glucose as they did from carbon 6. The ratio C-1^{14}/C-6^{14} gradually became smaller as the time of incubation increased. Even after 5 hours, when oxygen uptake was practically stopped, the ratio was still greater than 2. This showed, beyond question, that in 6-hour-old cells there was a definite preferential release of carbon 1 over carbon 6 of glucose as carbon dioxide. This condition remained, although it became decreasingly pronounced, to the stage where the cells had almost ceased to oxidize the substrates. The same general trend existed with 18-hour-old cells; although the ratio was only 7 after 30 minutes of incubation, it was still above 2 after 5 hours.

The results of a third experiment showed that the C-1^{14}/C-6^{14} for 36-hour-old cells was 4.38 at the end of 30 minutes, which is decidedly less than that obtained after the same incubation time with younger cells. Moreover, the ratios decreased to values less than 2 within and after 2 hours, whereas those of the 6- and 18-hour-old cells remained above 2 for the 5 hours of the experiment. These results indicate that resting 36-hour-old cells still preferentially

TABLE II

RADIOACTIVE CARBON DIOXIDE PRODUCED BY *P. pullulans*

	Average number of counts per minute, oxygen uptake, and $C\text{-}1^{14}/C\text{-}6^{14}$ for various incubation intervals									
	½ hour		1 hour		2 hours		3 hours		5 hours	
Sugar	c.p.m.	µl. O_2	c.p.m.	µl. O_2	c.p.m.	µl. O_2	c.p.m.	µl. O_2	c.p.m.	µl. O_2
				6-hour-old cells, 15.6 mg. dry weight						
Glucose-1-C^{14}	42	58	153	242	299	393	415	490	517	600
Glucose-6-C^{14}	3	60	27	250	60	410	130	510	234	620
Ratios	14.00		5.66		4.98		3.19		2.21	
				18-hour-old cells, 18 mg. dry weight						
Glucose-1-C^{14}	84	272	353	449	453	670	500	848	592	1036
Glucose-6-C^{14}	12	281	89	457	122	685	171	862	280	1060
Ratios	7.00		3.97		3.71		2.92		2.11	
				36-hour-old cells, 17.1 mg. dry weight						
Glucose-1-C^{14}	70	200	160	306	340	427	500	592	559	820
Glucose-6-C^{14}	16	205	50	310	223	440	432	600	531	821
Ratios	4.38		3.2		1.52		1.13		1.05	
				108-hour-old cells, 20 mg. dry weight						
Glucose-1-C^{14}	22	84	66	205	327	525	476	800	558	1006
Glucose-6-C^{14}	5	90	22	203	221	518	389	787	541	992
Ratios	4.40		3.14		1.48		1.22		1.03	

release carbon 1 of glucose over carbon 6 in the early stages of incubation, and that the tendency to release the sixth carbon atom of glucose as carbon dioxide is greater at this age than it is when the cells are 6 or 18 hours old. The results of a similar experiment with 108-hour-old cells show that the ratios are almost identical with those presented for 36-hour-old cells. This strongly indicates that after cells of *P. pullulans* are 36 hours old (or possibly less) their ability to release carbon dioxide from carbon 6 of glucose is not further increased with age.

The results of the experiments described above suggested that samples collected sooner than 30 minutes might give higher ratios. An experiment was done with a heavy suspension of 36-hour-old cells in which samples were taken after 10, 25, and 50 minutes of incubation. The results, presented in Table III, show that the ratio was 6 after 10 minutes. This is higher than it was after 25 or 50 minutes for cells at this age. It is, however, less than the values obtained after 30 minutes for 6- and 18-hour-old cells. As the cells were not aerated before they were pipetted into reaction-vessels, the endogenous respiration was so great in this particular experiment that, based on oxygen uptake figures, one would conclude that glucose was not utilized. The presence of radioactive carbon in the center wells of the reaction-vessels, however, proved that some of the sugar was metabolized. This fact also demonstrated conclusively that endogenous respiration is not inhibited during the oxidation of glucose.

TABLE III

RADIOACTIVE CARBON DIOXIDE PRODUCED IN 10, 25, AND 50 MINUTES BY 36-HOUR-OLD CELLS OF *P. pullulans**

	Average number of counts per minute, oxygen uptake, and $C\text{-}1^{14}/C\text{-}6^{14}$ ratios for the times of incubation designated					
	10 minutes		25 minutes		50 minutes	
Sugar	c.p.m.	$\mu l.\ O_2$	c.p.m.	$\mu l.\ O_2$	c.p.m.	$\mu l.\ O_2$
Glucose-1-C^{14}	6	0	32	0	88	0
Glucose-6-C^{14}	0	0	7	0	27	0
$C\text{-}1^{14}/C\text{-}6^{14}$	6.00		4.57		3.26	

*Dry weight, 50 mg.

III. METABOLISM OF GLUCOSE BY CELL-FREE EXTRACTS

The foregoing work indicated the possibility that very young cells of *P. pullulans* may metabolize glucose through the reactions of the pentose phosphate cycle, and that older cells may do so through a combination of the reactions of the pentose phosphate cycle and those of the Embden–Meyerhof scheme, but with greater use of the former than of the latter. Further work was required to confirm those indications. Preliminary experiments showed that oxygen consumption for resting cells was not greater than that of endogenous metabolism when glucose-1-phosphate, glucose-6-phosphate, ribose-5-phosphate, and fructose-1,6-diphosphate, respectively, were used as substrates. It was considered that the cells were impermeable to those compounds and that further elucidation of the intermediate metabolism of *P. pullulans* would be obtained by studying the activity of cell-free extracts. The purpose of the work reported in this section, therefore, was to determine if cell-free extracts of the organism would reduce triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN) in the presence of glucose, glucose-1-phosphate, and fructose-1,6-diphosphate, respectively; and to determine also if the extracts would oxidize ribose and ribose-5-phosphate.

Methods

Pullularia pullulans was grown for 36 hours in 2-liter Erlenmeyer flasks with strong aeration in the medium described in Section II. The cells were recovered with a Sharples centrifuge and resuspended in 2 liters of distilled water. The suspension was stirred for 30 minutes by means of a magnetic stirrer and again separated in the Sharples centrifuge. The cells were similarly washed and collected twice more and then stored at 5° C. until required. Cell-free extracts were prepared by grinding with alumina as described by McIlwain (9); the cell debris was removed by centrifuging at 0° C. for 1 hour at 6000 r.p.m., and the clear, slightly amber-colored liquid was stored in an ice bath until needed. Micro-Kjeldahl determinations were made to estimate the total protein.

Glucose-6-phosphate, fructose-1,6-diphosphate, and ribose-5-phosphate were purchased as barium salts and converted to sodium salts with Dowex 50. The Dowex 50 was regenerated by placing 50 g. on a filter paper in a small Buchner funnel and washing it slowly for 1 hour with a 4% aqueous solution of sodium hydroxide. The excess alkali was removed by washing with distilled water until the effluent reached pH 7; the resin was then treated with 2 *N* hydrochloric acid for 1½ hours, followed by washing with distilled water until the effluent was free of chlorine ions. This base–acid treatment was repeated four times and the resin was stored in the acid form. Twenty grams of the treated Dowex 50 were mixed with 50 ml. of distilled water and 1 g. of one of the respective phosphate compounds. The mixture was stirred for 5 hours and then filtered; the filtrate was neutralized with carbonate-free sodium hydroxide and dried under vacuum.

The enzymatic reductions of DPN and TPN were determined at room temperature by measuring increasing absorption at a wavelength of 340 mμ in a model DU Beckman spectrophotometer. Pentose was determined by Meijbaum's method as described by Umbreit *et al.* (13). The conditions maintained for each experiment are noted with the caption of the appropriate figure.

Results

Fig. 2 shows that cell extracts of *P. pullulans* rapidly reduce TPN in the presence of glucose-1-phosphate. Since TPN is linked with glucose-6-phosphate in known oxidations of glucose, it is concluded that the extract contained an active phosphoglucomutase to convert glucose-1-phosphate to glucose-6-phosphate. When DPN was used instead of TPN an increase of light absorption at 340 mμ did not occur. Neither TPN nor DPN were reduced when glucose was used instead of glucose-1-phosphate, which is evidence that the hexokinase of the cells was destroyed during the preparation of the extracts.

There was evidence of the presence of glucose-6-phosphate dehydrogenase in cell extracts. Fig. 3 shows that TPN was reduced very rapidly with glucose-6-phosphate as the substrate, but very slowly in the absence of that compound; it also shows that DPN was not reduced as rapidly as was TPN.

Fig. 4 shows that extracts of *P. pullulans* had the necessary enzymes to utilize ribose-5-phosphate rapidly. It was calculated that 106 μg. of ribose-5-phosphate were utilized in 20 minutes, under the conditions of the experiment. The extracts did not utilize non-phosphorylated ribose, which indicated that they did not contain ribokinase. When the extracts were heated to 100° C. and cooled, their ability to utilize ribose-5-phosphate was destroyed.

Fig. 5 presents evidence that usually denotes the existence of 3-phosphoglyceraldehyde dehydrogenase (3, 12). In this instance the enzyme caused a more rapid reduction of TPN than of DPN; it must, therefore, be linked with the former compound rather than with the latter. It may also be concluded from the evidence in Fig. 5 that the extracts contained an active fructose diphosphate aldolase.

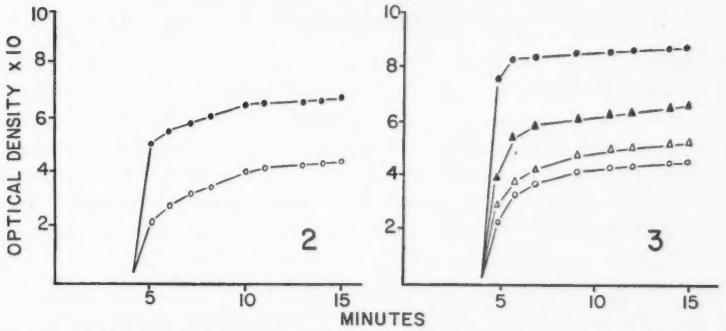

FIG. 2. Evidence for the presence of phosphoglucomutase in extracts of 36-hour-old cells of *P. pullulans*. ● TPN with glucose-1-phosphate; ○ TPN without glucose-1-phosphate. Test cuvettes contained 30 μM. of glucose-1-phosphate, 1.2 μM. of TPN and 1 ml. of cell extract (total nitrogen, 1.2 mg.). TPN added 4 minutes after start of experiment. Final concentration of phosphate buffer, 0.008 *M* (pH 7.2); of $MgCl_2.6H_2O$, 0.0099 *M*. Control cuvettes contained all components except glucose-1-phosphate. Final volume, 3 ml.

FIG. 3. Evidence for the presence of glucose-6-phosphate dehydrogenase in extracts of 36-hour-old cells of *P. pullulans*. ● TPN with glucose-6-phosphate; ○ TPN without glucose-6-phosphate; ▲ DPN with glucose-6-phosphate; △ DPN without glucose-6-phosphate. Test cuvettes contained 30 μM. of glucose-6-phosphate, 1.2 μM. TPN or DPN and 1 ml. of cell extract (total nitrogen for TPN experiment, 1.2 mg.; for DPN, 1.3 mg.). TPN or DPN added 4 minutes after start of experiment. Final concentration of phosphate buffer 0.008 *M* (pH 7.2); of $MgCl_2.6H_2O$, 0.0099 *M*. Control cuvettes contained all components except glucose-6-phosphate. Final volume, 3 ml.

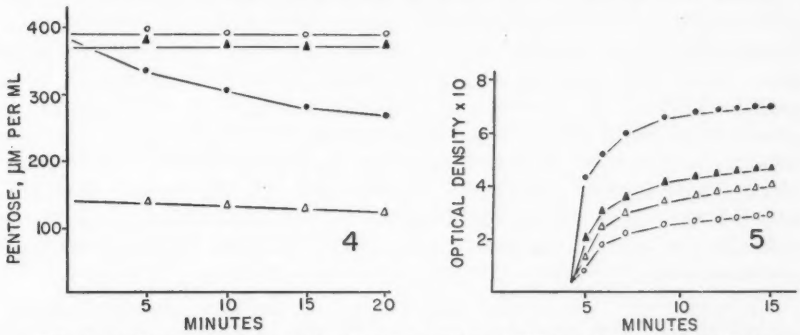

FIG. 4. Utilization of ribose-5-phosphate by extracts of 36-hour-old cells of *P. pullulans*. ○ Ribose+extract; ▲ ribose-5-phosphate+heated extract; ● ribose-5-phosphate + extract; △ extract. Tests were done in large tubes (25 mm. in diameter) at room temperature. Test tubes contained 10 ml. of cell extract (total nitrogen, 3.0 mg./ml.) and 1 ml. of solution of either ribose (3000 μg. per ml.) or ribose-5-phosphate (3000 μg. per ml.). Control tubes contained all components except ribose or ribose-5-phosphate. One ml. aliquots were removed at the times indicated, deproteinized with 5% trichloracetic acid, and analyzed for pentose. Final concentration of phosphate buffer, 0.008 *M* (pH 7.2) of $MgCl_2.6H_2O$, 0.0099 *M*. Total volume in all tubes, 11 ml.

FIG. 5. Evidence for the presence of D-glyceraldehyde-3-phosphate dehydrogenase in extracts of 36-hour-old cells of *P. pullulans*. ● TPN with fructose-1,6-diphosphate; ○ TPN without fructose-1,6-diphosphate; ▲ DPN with fructose-1,6-diphosphate; △ DPN without fructose-1,6-diphosphate. Test cuvettes contained 30 μM. of fructose-1,6-diphosphate, 1.2 μM. of either TPN or DPN, and 1 ml. of cell extract (total nitrogen, for TPN experiment, 1.1 mg.; for DPN experiment, 1.2 mg.). TPN or DPN added 4 minutes after start of experiment. Final concentration of phosphate buffer, 0.008 *M* (pH 7.2); of $MgCl_2.6H_2O$, 0.0099 *M*. Control cuvettes contained all components except fructose-1,6-diphosphate. Final volume, 3 ml.

Discussion

The inability of *P. pullulans* to utilize lactose is noteworthy in comparison with the findings of other workers. Fraser (5) studied the food requirements of one strain of the organism and reported that it utilized lactose slowly. Negroni and Fischer (10) studied the physiological characteristics of 30 strains and stated that they all utilized lactose as a carbon source. Wynne and Gott (14) tested the ability of 10 strains to ferment sugars and found that none fermented lactose; they did not state, however, if those strains were capable of using lactose as a source of carbon.

Under the conditions used in the present study, *P. pullulans* possessed constitutive enzymes for the utilization of all the sugars except lactose, since there was not an initial lag in the rate of oxygen uptake. Rhoades (11) obtained similar results during part of his study of the adaptive and constitutive enzymes of yeasts. He used nine strains of fermenting yeasts and found that all of them contained constitutive enzymes for the fermentation of glucose, mannose, sucrose, and raffinose regardless of the carbon source used in the original growth medium. He also found that the maltozymases in four of the strains were adaptive, but that this was only true when the cells were grown with carbon sources other than maltose, alpha-methylglucoside, and trehalose. Rhoades also found that galactozymase was adaptive in all but one of the nine yeasts. From Rhoades' findings it is important to note the variation that apparently can exist between similar microorganisms with regard to the activity of their enzymes. He found that every sugar tested was fermented by a constitutive enzyme system in at least one strain of yeast when cells of that yeast were grown on many or only certain sugars. It is possible, therefore, that a strain of *P. pullulans* other than that used in the present study might possess adaptive enzymes for one or more sugars. No less likely is the possibility that certain growth conditions could be found under which strain No. 11942 ATCC would exhibit some adaptive enzymic responses. Based on the results obtained in the present work, however, the conclusion is that this strain of *P. pullulans* does not have adaptive responses that are sometimes shown by other microorganisms.

The results show clearly that young cells of *P. pullulans* produce a greater amount of carbon dioxide from carbon 1 than from carbon 6 of glucose. This indicates that young cells metabolize glucose in such a manner that carbon 1 is the first to be released in the form of carbon dioxide. If living cells of any origin metabolize glucose only through the intermediates of the Embden–Meyerhof scheme, the carbon dioxide released would be derived from carbon 6 as rapidly as from carbon 1, for these will both become the methyl carbons of the pyruvate derivatives. Regardless of the manner by which pyruvate is further degraded, as many carbon dioxide molecules are produced from carbon 6 as from carbon 1 of glucose. If such a situation occurred in the present work, then $C\text{-}1^{14}/C\text{-}6^{14}$ should have equaled 1. Such a ratio was never obtained, although for 36- and 108-hour-old cells, it was 1.05 and 1.03, respectively, after 5 hours of incubation. Thus, although the possibility of the

existence of the classical glycolytic scheme of glucose metabolism in cells of *P. pullulans* cannot be eliminated, especially with cells older than 30 hours, another way of glucose dissimilation must occur; this could be through the reactions of the pentose phosphate cycle.

Studies with cell extracts of *P. pullulans* indicate that the organism is capable of catabolizing glucose by reactions similar to those of the Embden–Meyerhof system as well as by those of the pentose phosphate cycle. There is evidence of the presence of three enzymes of the glycolytic scheme, namely, phosphoglucomutase, fructose diphosphate aldolase, and 3-phosphoglyceraldehyde dehydrogenase. The reduction of TPN in the presence of glucose-6-phosphate and the apparent utilization of ribose-5-phosphate indicate the presence of at least two enzymes of the pentose phosphate cycle.

The possibility that an organism may catabolize glucose through the intermediates of both the glycolytic and pentose phosphate scheme may depend on the formation of fructose-1,6-diphosphate as an intermediate in the conversion of triosephosphate units to fructose-6-phosphate (6). Consequently the conversion of fructose-1,6-diphosphate to fructose-6-phosphate would take place by the activity of either 6-phosphofructokinase or the combined action of fructose diphosphate aldolase and transaldolase. Isomeration of the fructose-6-phosphate to glucose-6-phosphate would lead subsequently to a reduction of TPN by the activity of the TPN-linked glucose-6-phosphate dehydrogenase. If a series of reactions such as this occurred rapidly enough in cell extracts, they would surely cause a reduction of TPN in the presence of fructose-1,6-diphosphate (Fig. 4). Evidence for this contention has also been presented by Horecker *et al.* (7) from studies of the metabolism of rat liver.

In the reactions of the glycolytic scheme it has been well established that the 3-phosphoglyceraldehyde dehydrogenase of various microorganisms is DPN-linked. Cell extracts of *P. pullulans*, however, as well as those of *Penicillium chrysogenum* (12) preferentially reduce TPN in the presence of fructose-1,6-diphosphate. The 3-phosphoglyceraldehyde dehydrogenase in the microorganisms is therefore TPN-linked, or alternatively, the reduction of TPN is occurring at another reaction. The latter possibility could happen if the fructose-1,6-diphosphate is converted to glucose-6-phosphate, which should then be oxidized by the TPN-linked glucose-6-phosphate dehydrogenase. Further work is required to obtain more precise information about the intermediate metabolism of *P. pullulans*.

Acknowledgments

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THE EFFECT OF PASTEURIZATION ON LISTERIA MONOCYTOGENES[1]

RONALD E. BEARNS AND KENNETH F. GIRARD

Abstract

Using the so-called holding technique of pasteurization (61.7° C., 35 minutes) it was shown that when known numbers of viable *Listeria monocytogenes* are added to samples of fresh sterile milk, organisms may be recovered whenever the initial number of bacteria added is 5×10^4/ml. or greater. No significant differences in heat resistance among representatives of various *Listeria monocytogenes* was observed. After 48 hours at room temperature (22° C.) survivors multiply in such "pasteurized" milk and viable bacterial counts reach 10^8 per ml. without producing any grossly detectable changes in the milk or suspicious odors.

Introduction

It has been reported that *Listeria monocytogenes* may survive 5 minutes at 80° C. (10), 15 seconds at 100° C. (8), and pasteurization (9, 15). Information has not been found on the exact experimental methods used and the relationship, if any, of initial to surviving numbers of viable bacteria.

Data concerning the relative heat resistance of the various *L. monocytogenes* serotypes, which might account for the seemingly greater number of serotype 4*b* strains isolated from human cases (2), also appears to be lacking.

Materials and Methods

Six strains of *L. monocytogenes* were used in these experiments consisting of *L. monocytogenes* serotypes 1, 2, 3, 4*a*, 4*b* (13). Each *L. monocytogenes* strain was inoculated on to a Difco tryptose agar slope and incubated at 37° C. for 18–24 hours. The growth from each slope was suspended in distilled water and aliquots of the suspensions were adjusted by dilution to yield 5×10^8 viable organisms per ml. For each strain a series of nine screw-capped test tubes (20×150 mm.) in duplicate containing 9 ml. of sterile skim milk was inoculated with serial dilutions of the standard suspensions from 1:10–1:10,000,000. The ninth tube was not inoculated and served as a control. The milk used was pasteurized skim milk obtained from a local milk supplier. This milk was divided into aliquots of 300 ml. and placed in screw-capped quart bottles. The bottles were autoclaved at 115° C. for 15 minutes, cooled in the refrigerator at 4° C. for 2 to 3 hours, then placed in a water bath, and maintained at 62° C. for 35 minutes. Tests on this milk showed the samples to be sterile.

Following addition of *L. monocytogenes* to the sterile milk samples, these were then pasteurized in a constant temperature water bath which brought the temperature of the milk in the tubes to 61.7° C. within 2 minutes. The milk surface in the tubes was kept 3 to 4 cm. below the water level in the bath

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Contribution from The Department of Bacteriology and Immunology, McGill University, Montreal, Que.

and during the entire course of the experiment the tubes were agitated. At the end of the pasteurization period the number of survivors was determined by a modification of the drop plate method (12). The sample was thoroughly mixed and 0.1 ml. was pipetted onto the surface of a Difco tryptose agar plate. The fluid added was spread over the agar surface by gently tilting the plate and after drying was incubated at 37° C. for 48 hours. Colony counts made at this time compared closely with drop plate and pour plate results on the same samples.

The water bath (temperature 61.7° C. ± 0.5° C.) used for pasteurization was covered with a sloping plexiglass-topped hood having a slot in one end. A Boerner shaker with an attached horizontal aluminum bar was placed end to end with the water bath so that the bar entered the slot in one end of the hood. A wire test tube holder was suspended from the bar inside the hood so that it was immersed in the water and would agitate freely. The tubes containing the milk samples were allowed to "bounce" in the holder to aid in the dispersion of the organisms throughout the milk samples. A control sample containing a specially fitted thermometer showed that the temperature of the milk sample inside the tubes was the same as that of the water outside the tubes ± 0.5° C. over the pasteurization period.

Experimental Results

Counts were given in Table I for all *L. monocytogenes* strains tested. Results were also given in terms of the decimal reduction time (DRT) described by Katzin *et al.* (5), which is defined by the author as follows:

$$\text{DRT} = 2.3/K = (T_2 - T_1)/\log_{10}(C_1/C_2)$$

where K is the monomolecular reaction rate constant, and C_1 and C_2 are the initial and final concentrations of bacteria respectively subjected to a constant lethal temperature for $(T_2 - T_1)$ minutes.

This is based upon the application of the monomolecular reaction rate constant to bacterial death under uniform conditions. For example, milk having an initial concentration of *L. monocytogenes* of 5×10^6/ml. contains 3×10^3/ml. after 35 minutes at 61.7° C.

$$\log_{10}(C_1/C_2) = \log_{10}(5{,}000{,}000/3000) = 3.22$$

$$\text{DRT}_{61.7°} = 35/3.22 = 10.9 \text{ minutes.}$$

This means that after 10.9 minutes 10^1 organisms have been killed, after 21.8 minutes 10^2, after 32.7 minutes 10^3, etc. Extending this example one may calculate that more than 1667 viable *L. monocytogenes* per ml. are necessary in order to obtain survivors after 35 minutes at 61.7° C. Actually, under the experimental conditions employed, from 5000 to 50,000 *L. monocytogenes* per ml. was found to be the limiting initial concentration (Table I).

In the report by Katzin *et al.* (5) of 13 coliform strains, the highest DRT obtained was 1.3 and of the many non-spore formers examined by this method only one, that is certain of the Group D streptococci, displayed the same order of heat resistance as do the *Listeria*.

TABLE I

DECIMAL REDUCTION TIME VALUES AT VARIOUS INITIAL CONCENTRATIONS

Initial	Survivors	DRT	Initial	Survivors	DRT
LM serotype 1, strain 9-127			LM serotype 1, strain 9-138		
5×10^{7}	3×10^{4}	10.8	4×10^{7}	2×10^{4}	10.6
5×10^{6}	3×10^{3}	10.8	4×10^{6}	2×10^{3}	10.6
5×10^{5}	3×10^{2}	10.8	4×10^{5}	2×10^{2}	10.6
5×10^{4}	3×10^{1}	10.8	4×10^{4}	1.5×10^{1}	10.2
5×10^{3}	None	–	4×10^{3}	None	–
5×10^{2}	None	–	4×10^{2}	None	–
5×10^{1}	None	–	4×10^{1}	None	–
LM serotype 2, strain 9-128			LM serotype 3, strain 9-63		
5×10^{7}	3×10^{4}	10.8	5×10^{7}	2×10^{4}	10.3
5×10^{6}	3×10^{3}	10.8	5×10^{6}	2×10^{3}	10.3
5×10^{5}	3×10^{2}	10.8	5×10^{5}	2.7×10^{2}	10.7
5×10^{4}	3×10^{1}	10.8	5×10^{4}	2.5×10^{1}	10.6
5×10^{3}	None	–	5×10^{3}	None	–
5×10^{2}	None	–	5×10^{2}	None	–
5×10^{1}	None	–	5×10^{1}	None	–
LM serotype 4*a*, strain 9-71			LM serotype 4*b*, strain 9-137		
5×10^{7}	2.5×10^{4}	10.6	5×10^{7}	1×10^{4}	9.5
5×10^{6}	2.5×10^{3}	10.6	5×10^{6}	1×10^{3}	9.5
5×10^{5}	2.5×10^{2}	10.6	5×10^{5}	1×10^{2}	9.5
5×10^{4}	2.5×10^{1}	10.6	5×10^{4}	1×10^{1}	9.5
5×10^{3}	None	–	5×10^{3}	None	–
5×10^{2}	None	–	5×10^{2}	None	–
5×10^{1}	None	–	5×10^{1}	None	–

Since *L. monocytogenes* exhibited a comparatively high heat tolerance it seemed of some value to determine how well this organism might survive or multiply in such milk specimens upon holding at room temperature.

Pasteurized skim milk was obtained from a local milk supplier and separated into two aliquots. One was sterilized as in the previous experiment, the other was not. Both were inoculated with *L. monocytogenes* serotype 4*b*, strain 9-137 of human origin, to a concentration of less than 1000 bacteria per 500 ml. of milk. These flasks were then allowed to incubate at room temperature (22° C.) for 48 hours.

The sterile milk samples to which *L. monocytogenes* was added resulted in pure cultures of 4×10^{8}/ml. The appearance of the milk was unchanged, and there was no detectably different odor or consistency. (It was not tasted.)

The dairy-pasteurized but non-sterile samples that had been inoculated with *L. monocytogenes* resulted in mixed cultures and it was impossible to determine accurately the degree of *L. monocytogenes* multiplication. In addition the milk had curdled and soured. Previous, as yet unpublished, experiments (6) as shown in Fig. 1 depict the growth curve of *L. monocytogenes* at 6° C. compared to those of *Escherichia coli* and *Micrococcus pyogenes*. Although these counts were carried out using tryptose broth as a medium there

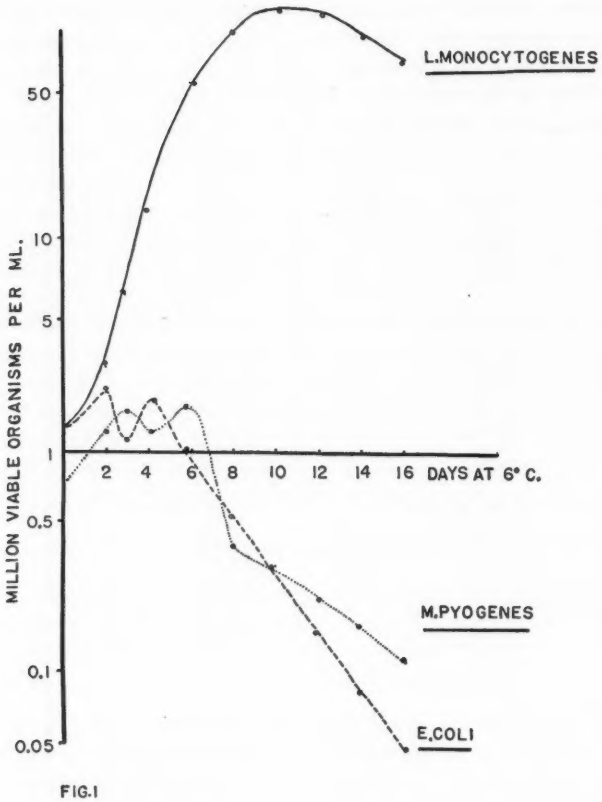


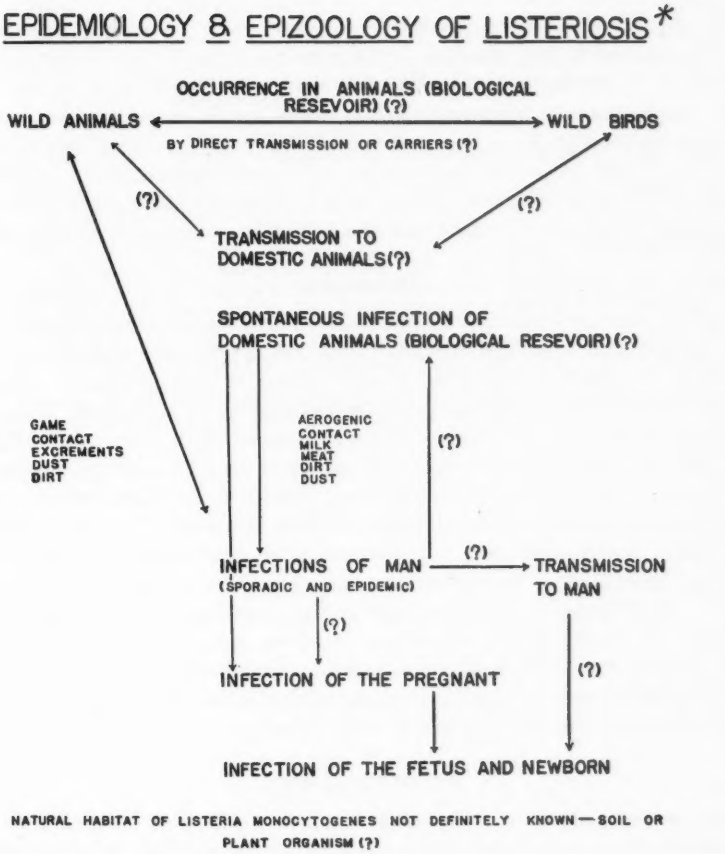
FIG.1

TABLE II

HEAT RESISTANCE OF *Listeria monocytogenes* IN MILK AND WATER

Before past.*	After past.	Before past.	After past.
LM strain 9-137 in milk		LM strain 9-137 in water	
5×10^7	1×10^4	5×10^7	1×10^4
5×10^6	1×10^3	5×10^6	1.5×10^3
5×10^5	1×10^2	5×10^5	1×10^2
5×10^4	1×10^1	5×10^4	1×10^1
5×10^3	None	5×10^3	None
5×10^2	None	5×10^2	None
5×10^1	None	5×10^1	None

*61.7° C., 35 minutes.



* FROM: SEELIGER, H.P.R., LISTERIOSE, J.A. BARTH, LEIPZIG, 1955.
(ADJUSTED FOR 2ND ED. TO BE PUBLISHED AND REPRODUCED BY PERMISSION OF THE AUTHOR).

FIG. 2

is little reason to expect the *L. monocytogenes* growth curve to differ widely from that in milk at this temperature. As can be seen from the graph the peak of the log phase of growth is reached in about 10–11 days at 6° C. There is then the possibility of *L. monocytogenes* multiplication even in refrigerated milk prior to the discard of such milk for purely aesthetic reasons. This assumes a measure of contamination at source prior to or after pasteurization that is purely hypothetical at this time since very little is known of the ecology of *L. monocytogenes* or whether it actually occurs in bulk raw milk with any frequency. Since the DRT values for *L. monocytogenes* in milk were comparatively high the experiment was repeated using sterile water instead of milk to determine whether milk itself exercised any protective effect. Results showing heat resistance of *L. monocytogenes* in milk and water are given in Table II. In each case the end point is approximately the same.

Discussion

The recent experiments of Gray and his colleagues (3, 4) have emphasized the oral route as a possible avenue of infection in pregnant rabbits and goats resulting in abortion, stillbirth, or early death of the newborn. The apparently greater heat tolerances of *L. monocytogenes* to most other non-spore forming, potentially pathogenic, bacteria coupled with the ability of this organism to grow at refrigeration temperatures as shown in this report are properties of some importance when one considers that listeriosis enjoys a widespread geographical distribution and host spectrum (7). It is perhaps significant that the disease is not uncommon amongst ruminant animals (14) and that infections of the udder have been described in cattle (1). In addition, *L. monocytogenes* has been isolated from raw milk samples (11, 16) in Europe. It would appear, therefore, that the possibility of contaminated milk, whether raw or pasteurized, as indicated by Seeliger (13) (Fig. 2) serving as one possible source of infection cannot be overlooked in any epidemiological investigation of listeriosis especially in the newborn.

Acknowledgment

We are indebted to the National Research Council of Canada for their support of these and other studies related to the problem of listeriosis in animals and man.

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NOTES

APHIDS REMAIN INFECTIVE WITH TURNIP-LATENT VIRUS AFTER A MOLT[1]

J. P. MacKinnon and R. H. E. Bradley

Only recently Day (1) pointed out what should have long been obvious: the importance of showing if insect vectors of viruses remain infective after they have molted. If they do, it is good evidence that the virus is not carried by the stylets, because during a molt the stylets are shed and new ones are formed from within the head capsule. But he found that this information is not available for many viruses, particularly aphid-borne viruses. The information should be given with each new report of an insect-borne virus.

MacKinnon (2) reported a turnip-latent virus that is transmitted by the aphid *Myzus persicae* (Sulz.). Though symptomless or nearly so in turnip, the virus causes pronounced systemic symptoms in *Physalis floridana* Rydb. (2). Therefore this host was used both as a virus source and as a test plant in the following experiments to show if *M. persicae* remains infective with turnip-latent virus after the aphid has molted.

Several adult aphids were placed for a day or two on an infected plant, and young born during this time were left to feed on the infected plant until they were about half-grown (about 4 days after the adults were removed). These half-grown aphids were transferred singly to young healthy test plants, and thence to a new test plant each day thereafter. During these daily transfers, records were kept of any cast aphid skins found, which showed that the aphid molted. Of 29 aphids so tested, 23 transmitted the virus. These 23 infective aphids survived from 2 to 15 transfers without any indication that molting affected their infectivity. Transmissions appeared to be less likely on the day an aphid died or the day or two before. Table I shows the results of the first 10 transfers of aphids that lived this long or longer; most of these aphids molted two or three times; none failed to transmit after a molt. Table I also shows that aphids were much more apt to transmit on days that they molted than on those when they did not. This fact may be worthy of further study.

Half-grown aphids were also transferred daily to test plants after each spent only 6 hours on an infected plant. Though fewer aphids transmitted the virus, those that did transmit did so as readily after molting as before.

In these experiments, the aphids usually matured and had young after the fifth or sixth transfer. These young were placed on healthy plants and allowed to remain for several days. Over 150 young from infective adults were so tested, but none transmitted the virus. Thus turnip-latent virus

[1]Issued as a joint contribution from the Botany and Plant Pathology Division (No. 1635) and the Entomology Division (No. 3682), Science Service, Canada Department of Agriculture, Ottawa, Ontario.

TABLE I

PLANTS INFECTED BY SINGLE APHIDS THAT FED ON A DIFFERENT *P. floridana* SEEDLING EACH DAY FOR 10 DAYS

(Aphids born on infected plant and fed thereon about 4 days before transfers began)

Aphid No.	Day of transfer									
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
1	+	+*	−	+	+*	+	+	+	+	+
2	−	+*	+*	+*	+	−	+	−	−	−
3	+	+*	+	+*	+*	+	−	+	+	+
4	−	+	+*	+	+*	+	−	+	+	+
5	+*	−	+	+*	−	+*	−	+	−	−
6	−	+	+*	−	+*	+*	+	+	+	+
7	−	+	+*	+	+*	+*	−	+	+	+
8	−	+*	+	+	+*	−	−	+	−	−
9	−	+*	+	−	+*	−	−	+	+	+
10	−	−	−	+	+*	−	+	−	−	−

NOTE: + = Plant infected.
− = Plant not infected.
* = Cast skin found, which shows aphid molted that day.

is rarely if ever passed from infective aphids to their young. And as these young fed up to 24 hours on newly infected plants, apparently aphids do not ingest enough virus to become infective during the first day following infection of a plant. Nymphs born on plants infected over 3 weeks ingested enough virus within a few hours so that they later became infective.

Hence, *M. persicae* after it has molted remains infective with turnip-latent virus. Presumably it is a virus like several others, including the potato leaf roll virus, that undergoes some internal passage through the vector and is then reinjected into plants on which the aphid subsequently feeds.

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Manuscripts, in English or French, should be typewritten, double spaced, on paper 8½ × 11 in. **The original and one copy are to be submitted.** Tables and captions for the figures should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered.

Style, arrangement, spelling, and abbreviations should conform to the usage of recent numbers of this journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed.

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Tables should be numbered in roman numerals and each table referred to in the text. Titles should always be given but should be brief; column headings should be brief and descriptive matter in the tables confined to a minimum. Vertical rules should not be used. Numerous small tables should be avoided.

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